HORMONE ACTION AND SIGNAL TRANSDUCTION IN ENDOCRINE PHYSIOLOGY AND DISEASE

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-100-8
DOI 10.3389/978-2-88966-100-8

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HORMONE ACTION AND SIGNAL TRANSDUCTION IN ENDOCRINE PHYSIOLOGY AND DISEASE

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Citation: Hunyady, L., Balla, T., eds. (2020). Hormone Action and Signal Transduction in Endocrine Physiology and Disease. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-100-8

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frontiers in Endocrinology

EDITORIAL
published: 27 August 2020
doi: 10.3389/fendo.2020.00589

Editorial: Hormone Action and Signal Transduction in Endocrine Physiology and Disease

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Keywords: signal transduction, ACTH, G protein, beta-arrestin, sertolli cells, gonadotroph cell, myometrium contractility, TSH receptor

Editorial on the Research Topic

Hormone Action and Signal Transduction in Endocrine Physiology and Disease

The Frontiers Research Topic on "Hormone Action and Signal Transduction in Endocrine Physiology and Disease" is a collection of 10 papers inspired by the meeting held at Semmelweis University Budapest, August 16-17, 2018 in memory of Kevin J. Catt who passed away on October 1, 2017. Dr. Catt was a notable scientist who worked in areas related to receptors of protein and peptide hormones and their actions via various signal transduction mechanisms in many tissues. His interest was focused on the hypothalamo-adrenal axis as well as on the hormonal control of reproductive organs. The collection of chapters authored by Dr. Catt's disciples who spent time under his mentorship reflects the legacy of Dr. Catt in these research areas. The Authors are well-established and highly accomplished scientists who work on receptors, their signaling modalities and on biological effects of hormones and neurotransmitters. Although not a former trainee, a close collaborator of Dr. Catt has also contributed to this collection.

The chapters cover endocrine related functions in several organs including hypothalamus, pituitary and adrenal gland, reproductive organs, fetal signals, and ovarian tumors. They summarize recent advances and new discoveries on the functions of hormones, their signaling pathways and their impact on physiology as well as on pathology of various neuro- and somatic diseases.

Four of the Chapters focus on the hypothalamo-adrenal stress regulatory axis.

The Chapter by Antoni gives a historical perspective on the role of vasopressinergic neurons located in the supraoptic and paraventricular nuclei in the control of ACTH secretion from the anterior pituitary. This review covers an important topic, namely conditions under which the feed-back inhibition by glucocorticoid hormones in pituitary corticotrophs is bypassed (termed glucocorticoid escape) and the role of vasopressinergic neurons in the process.

The Chapter by Parra-Mercado et al. from the Hauger and Olivares-Reyes laboratories investigates the signaling properties of the corticotropin releasing factor receptor (CRF1R) in heterologous expression systems. This work teases out the contribution of tyrosine kinases, such as Src and EGF receptors as well as the known antiapoptotic phosphatidylinositol 3-kinase (PI3K) pathways in the control of ERK1/2 phosphorylation, the main signaling hub of CRF1R activation.

The Chapter by Clark and Chan. covers ACTH receptors (MC2R) that initiate activation and signaling in the adrenal cortex. They highlight the importance of the adrenal accessory protein, MRAP, which is essential for MC2R expression and its trafficking to the cell surface. The review discusses the control of mRNA and protein expression of MC2R and MRAP by ACTH, as well as the role of N-glycosylation in the stabilization of the MRAP homodimer. The dynamic association

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 May 2020*
Accepted: *20 July 2020*
Published: *27 August 2020*

Citation:
Balla T and Hunyady L (2020) Editorial: Hormone Action and Signal Transduction in Endocrine Physiology and Disease. Front. Endocrinol. 11:589. doi: 10.3389/fendo.2020.00589

between MC2R and MRAP and its impact on ACTH receptor desensitization also receives ample coverage. The role of these processes in the control of adrenal zonation and cell renewal as they relate to adrenal diseases, cancer, and aging are discussed in detail.

The Chapter by Turu et al. from the group of László Hunyady focuses on the structure-function relationship of the AT1 angiotensin receptor, the main controller of mineralocorticoid secretion from the adrenal cortex. The authors discuss the structural features of the receptor that are critical for G protein coupling and activation as well as for β-arrestin binding during ligand engagement. They also highlight the fact that different ligands can induce conformational changes that selectively favor interaction of the receptor with their distinct protein binding partners mediating various aspects of their signaling. These findings offer new opportunities for pharmacological modulation of G protein and β-arrestin mediated signaling effects of G-protein coupled receptors independently.

Five chapters cover research areas related to the reproductive system. The central role of FSH, acting in concert with androgens in shaping pulsatile LH secretion is a major topic of interest. The effects of these hormones on Sertoli cell gene expression receive in-depth coverage and so does the Involvement of paracrine signals that are essential for the progress of spermatogenesis.

Bjelobaba et al. from the Stojilkovic group describe single cell RNA profiling studies from pituitary cells that revealed that gonadotroph cells display uniques expression patterns of osteopontin and its sibling, Dentin matrix protein 1. The authors discuss the implication of these factors in defining the tissue architecture of the anterior pituitary and its possible response to changes in the reproductive cycle and during tumorigenesis.

Meroni et al. from the Cigorraga group present an extensive overview on the hormones and locally produced factors as well as their signal transduction pathways and molecular mechanisms that control Sertoli cells proliferation and maturation. These data highlight mechanisms by which the number of Sertoli cells is controlled, which is an important determinant of the spermatogenic capacity of the testis. The review also discusses possible harmful effects caused by exposure to pollutants and frequently used pharmaceutical products in premature decrease in Sertoli cell number and impaired spermatogenesis.

Mendelson et al. review the factors that control myometrium contractility during pregnancy, with special emphasis on changes that contribute to the initiation of labor in humans and rodents. The authors discuss mechanisms by which progersterone, acting through various progesterone receptor isoforms, maintains myometrial quiescence during pregnancy. They also describe changes that underlie the decline in progesterone receptor function, leading to initiation of labor and the roles of miRNAs regulated by estradiol and progesterone in these processes.

Dufau and Kavarthapu describe the essential role of GRTH/DDX25, an RNA helicase, in the process of spermatogenesis. This helicase, discovered in the Dufau laboratory, is responsible for the elongation process of round spermatocytes. The biochemical events and various genes that are involved (mRNA transport/ translation) by the GRTH/DDX25 helicase receive ample coverage. The regulation of GRTH by androgens through an increases in the level of the transcription factor GCNF in round spermatids establishes a paracrine interplay between Sertoli cells and germ cells. Inhibition of the phosphorylation of GRTH is currently being pursued by this group as a potential orally active and reversible male contraceptive strategy.

Tocci et al. from the group of Bagnato review the importance of Endothelin-1 Receptor (ET-1R) and its signaling in the development of ovarian cancer and discuss new therapeutic approaches interfering with this system. They describe how ET-1R and β-arrestin-1 mediated signaling contribute to tumor progression in the ovary and describe combinatorial approaches as potential means to block these pathways.

Lastly, Latif et al. from the Davies laboratory describe recent progresses in developing small molecules that could stimulate TSH receptors, specifically engaging the Gq-phospholipase C pathway for pharmacological control of thyroid hormone secretion. They authors review the properties of a new agonist that activates cross-talk between the Ca^{2+} and cAMP messenger systems and discuss its potential use in controlling proliferation of thyroid cells.

The collection of these chapters not only honors the Dr. Catt and his scientific legacy but will undoubtedly inspire both new investigators and established scientists to advance these important research areas with their own research.

AUTHOR CONTRIBUTIONS

TB and LH contributed to the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 16 April 2019
doi: 10.3389/fendo.2019.00224

Molecular Mechanisms and Signaling Pathways Involved in Sertoli Cell Proliferation

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Sertoli cells are somatic cells present in seminiferous tubules which have essential roles in regulating spermatogenesis. Considering that each Sertoli cell is able to support a limited number of germ cells, the final number of Sertoli cells reached during the proliferative period determines sperm production capacity. Only immature Sertoli cells, which have not established the blood-testis barrier, proliferate. A number of hormonal cues regulate Sertoli cell proliferation. Among them, FSH, the insulin family of growth factors, activin, and cytokines action must be highlighted. It has been demonstrated that cAMP/PKA, ERK1/2, PI3K/Akt, and mTORC1/p70SK6 pathways are the main signal transduction pathways involved in Sertoli cell proliferation. Additionally, c-Myc and hypoxia inducible factor are transcription factors which participate in the induction by FSH of various genes of relevance in cell cycle progression. Cessation of proliferation is a pre-requisite to Sertoli cell maturation accompanied by the establishment of the blood-testis barrier. With respect to this barrier, the participation of androgens, estrogens, thyroid hormones, retinoic acid and opioids has been reported. Additionally, two central enzymes that are involved in sensing cell energy status have been associated with the suppression of Sertoli cell proliferation, namely AMPK and Sirtuin 1 (SIRT1). Among the molecular mechanisms involved in the cessation of proliferation and in the maturation of Sertoli cells, it is worth mentioning the up-regulation of the cell cycle inhibitors p21Cip1, p27Kip, and p19INK4, and of the gap junction protein connexin 43. A decrease in Sertoli cell proliferation due to administration of certain therapeutic drugs and exposure to xenobiotic agents before puberty has been experimentally demonstrated. This review focuses on the hormones, locally produced factors, signal transduction pathways, and molecular mechanisms controlling Sertoli cell proliferation and maturation. The comprehension of how the final number of Sertoli cells in adulthood is established constitutes a pre-requisite to understand the underlying causes responsible for the progressive decrease in sperm production that has been observed during the last 50 years in humans.

Keywords: Sertoli cells, proliferation, maturation, signal transduction, xenobiotics

OPEN ACCESS

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *05 February 2019*
Accepted: *21 March 2019*
Published: *16 April 2019*

Citation:
Meroni SB, Galardo MN, Rindone G, Gorga A, Riera MF and Cigorraga SB (2019) Molecular Mechanisms and Signaling Pathways Involved in Sertoli Cell Proliferation. Front. Endocrinol. 10:224. doi: 10.3389/fendo.2019.00224

INTRODUCTION

Sertoli cells represent one of the most complex cells in the organism. Not only because of their three-dimensional structure but also due to their function to create a unique environment which allows germ cell development. The concept of "nurse cells" is widely used to refer to this cell type as they create a complete lining within the tubular walls which envelope spermatogenic cells. They also, by virtue of tight junction formation, constitute the main component of the blood testis barrier (BTB).

Positive correlations between total Sertoli cell number and the daily sperm production in several species, including humans, have been reported (1–3). This relationship exists because each Sertoli cell is able to sustain a limited number of germ cells (4). Thus, it may be concluded that appropriate development of the Sertoli cell population, with respect to their number and functionality, will determine spermatogenic capacity through adulthood.

Only immature Sertoli cells proliferate and, even when there are differences between species as to the pre-dominant periods of mitotic activity, it is generally accepted that proliferation stops at puberty in most species (5). Thus, the regulation of Sertoli cell proliferation—determining the final Sertoli cell number—and cessation of the proliferation concomitant with maturation—establishing adequate cell function—constitutes the foundation of adult testicular function and occurs in fetal and early postnatal life.

From the initial studies of Sertoli (6) significant advances have been made in understanding the functionality of this cell type. It is well-known that the gonadotropin Follicle-Stimulating Hormone (FSH) and also androgens regulate the proliferation and functional maturation of this cell type. In addition to these classical hormones, a great number of locally produced factors participate in the regulation of Sertoli cells reflecting one of the most representative examples of cell-cell communication (7).

Noticeably, while a huge number of reports deal with the regulation of several aspects of Sertoli cell physiology and at least two books of biblical proportions have been published (8, 9), much less is known on the regulation of proliferation of this cell type. Clermont and Perey (10) performed the earliest quantitative study of Sertoli cell proliferation based on the analysis of the percentage of Sertoli cells undergoing mitosis. More than 10 years later, Steinberger and Steinberger (11) utilizing organ culture pulsed with [^{3}H]-thymidine and determining the labeling index showed that this index decreased with the age of the animal. A few years later, Griswold et al. (12) showed that FSH stimulates DNA synthesis and mitosis of immature Sertoli cells in culture and that mitotic activity is limited to immature cells. Furthermore, studies performed by Orth (13, 14) identified fetal and postnatal life in the rat as moments of high mitotic activity. From these initial studies that established the basic concepts on Sertoli cell proliferation a great number of investigators have tried to deeply understand the molecular mechanisms underlying this physiological process.

Increasing evidence for the quantitative and qualitative decline in human sperm over the past few decades has been presented (15–17). A recent study by Levine et al. (18) reported a 50–60% decline in sperm counts. The reasons for this decline are not clear yet, but modern lifestyle may be a cause (19–22). A variety of factors including those that affect Sertoli cell proliferation and maturation at cessation of mitosis might be somehow related to the impairment observed in seminiferous tubule function. Our review will highlight molecular mechanisms related to the above-mentioned processes, i.e., proliferation and maturation, which may be affected by exposure to certain therapeutic drugs or pollutants.

MAIN FACTORS INVOLVED IN THE STIMULATION OF SERTOLI CELL PROLIFERATION

Even though a considerable variation in the number of Sertoli cells among members of the same specie exists, it is assumed that the final number of Sertoli cells in adults results from events in fetal, neonatal or peripubertal life (23). Therefore, the hormones and locally produced factors as well as signaling pathways and molecular mechanisms involved in the stimulation of Sertoli cell proliferation are crucial to define sperm production in adult animals. Current knowledge of the roles FSH, the insulin family of growth factors, the activins and cytokines play in Sertoli cell proliferation will be summarized in the following sections.

Follicle-Stimulating Hormone

FSH is a gonadotropin synthesized and secreted by the gonadotropic cells of the anterior pituitary gland. FSH consists of two different glycoprotein subunits: a common α subunit, which is present in other pituitary hormones, and a specific β subunit, which confers its specific biologic action (24). FSH is a central regulator of reproductive function in mammals. Physiological effects of FSH are mediated by its association with the FSH receptor (FSHR), a seven-transmembrane-domain protein, which belongs to the G protein-coupled receptor (GPCR) superfamily (25, 26). It is widely accepted that FSHR in the male is expressed exclusively in Sertoli cells (27). Consistent with the presence of receptors, FSH actions can be demonstrated during fetal life and throughout postnatal lifespan. However, the physiological response to FSH varies depending on the state of maturation of Sertoli cells (5). FSH regulates Sertoli cell proliferation only during fetal and early postnatal life, whereas it regulates differentiation after cessation of mitosis at puberty. The first demonstration of a stimulatory role of FSH in the proliferation of Sertoli cells came from studies of Griswold et al. (12, 28, 29). Other pioneering studies that established the relevance of FSH in the regulation of Sertoli cell proliferation consisted of *in vivo* procedures that lead to diminished endogenous FSH levels -decapitation *in utero* or addition of FSH antiserum to rat fetuses. These experiments showed that, as a result of lower FSH levels, incorporation of [^{3}H]-thymidine in Sertoli cells decreased (14). In these studies, it was also shown that FSH increases the number of Sertoli cells in organ culture. In addition, it was shown that hemicastration of 3-day-old rats evokes enhanced Sertoli cell proliferation in the remaining testis that is accompanied by elevated levels of FSH,

and that testosterone administration abrogates the compensatory hypertrophy (30). This negative effect of testosterone on Sertoli cell proliferation was interpreted to be a consequence of the negative feedback on FSH secretion that testosterone exerts. The importance of FSH in the regulation of Sertoli cell proliferation was further confirmed by a study conducted by Almirón and Chemes (31). The latter authors observed that Sertoli cell mitotic index was reduced in immature rats with FSH withdrawal accomplished by administration of high doses of testosterone propionate, and that the index increased when FSH levels were restored by injection of human FSH. Years later, the results obtained utilizing gonadotropin-deficient hypogonadal (hpg) mice treated with recombinant FSH (32, 33) or hpg mice expressing transgenic FSH (34, 35) strengthened the role of FSH in the regulation of Sertoli cell proliferation. Complementarily, a reduction in Sertoli cell number in mice with a null mutation in *Fshr* gene was observed (36–38).

Once the mitogenic role of FSH was convincingly demonstrated, further studies focused on elucidating signal transduction pathways involved in the regulation of Sertoli cell proliferation triggered by the hormone. For more than 20 years, it had been widely accepted that the canonical Gs/cyclic adenosine monophosphate (cAMP)/cAMP-dependent kinase (PKA) pathway was the unique mechanism that contributed to FSH actions (39, 40). The increase in [^{3}H]-thymidine incorporation in immature Sertoli cells caused by dibutyryl-cAMP (dbcAMP) incubations (14, 29) was the first evidence for the participation of cAMP-dependent pathways in the regulation of Sertoli cell proliferation. Nowadays, growing evidence indicates the complexity associated with FSH-induced cellular signaling (41, 42). Crépieux et al. (43) showed that FSH activates the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway following dual coupling of the FSHR both to Gs and to Gi heterotrimeric proteins, in a PKA- and also Src-dependent manner, leading to cell cycle progression through cyclin D1 induction and the concomitant proliferation of Sertoli cells from immature rats. The complexity of the signaling network triggered by FSHR is also reflected by the activation of phosphatidyl-inositide-3 kinase (PI3K)/Akt/p70 S6 kinase (p70S6K) by FSH in proliferating Sertoli cells (44). More recently, Riera et al. (45) showed that FSH regulates proliferation through PI3K/Akt/mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. At the molecular level, an increase in phosphorylated (P)-Akt, P-mTOR, and P-p70S6K levels induced by FSH in proliferative Sertoli cells was observed. Additionally, FSH increased the levels of P-PRAS40, a substrate of Akt and a component of the mTORC1, probably contributing to improving mTORC1 signaling. Furthermore, the decrease in FSH-stimulated P-Akt, P-mTOR, P-p70S6K, and P-PRAS40 levels in the presence of a PI3K specific inhibitor emphasized the participation of PI3K in FSH signaling. Additionally, the inhibition of FSH-stimulated Sertoli cell proliferation by the effect of specific inhibitors of PI3K and mTOR confirmed the relevance of the PI3K/Akt/mTORC1 signaling pathway in the mitotic activity of FSH. These authors also showed that FSH decreased the levels of P-AMP-activated protein kinase (AMPK)—serine/threonine protein kinase that antagonizes mTORC1 actions—and concluded that AMPK-dependent mechanisms counteract FSH proliferative effects.

The intricate signaling actions of FSH are responsible for extensive alterations in the expression of genes due to the activation of a number of transcription factors in Sertoli cells. In addition to cAMP response element binding protein (CREB) (46), FSH modulates the transcriptional activity of: NFκB (47), AP1 (48), c-Myc (49), hypoxia-inducible factor (HIF)1 (50), and HIF2 (50, 51). The majority of the latter studies on transcription, with the exception of those performed on c-Myc and HIF2, were carried out in non-proliferative Sertoli cells and did not address whether these FSH-activated transcription factors might be involved in the regulation of Sertoli cell proliferation. Concerning c-Myc, it was observed that c-Myc mRNA is clearly detectable in Sertoli cells from 8-day-old rats but hardly detectable in cells from those aged 14 and 28 days. Besides, it was observed that FSH, in a cAMP-dependent manner, stimulates c-Myc mRNA expression in Sertoli cells derived from 8- to 14-day-old rats but has almost no effect in those derived from 28-day-old rats (49). This age-dependent regulation of expression strongly suggests a role of c-Myc in immature Sertoli cells. A cAMP-dependent pathway is not the only one regulating c-Myc expression as the involvement of PI3K signaling pathway has also been demonstrated (45). As for HIFs, it was shown that FSH upregulates the expression of HIF1α and HIF2α in Sertoli cells obtained from 20-day-old rats (50), a developmental stage in which rat Sertoli cells barely proliferate and have established the BTB. On the other hand, in Sertoli cells obtained from 8-day-old rats, a stage in which Sertoli cells are actively proliferating, FSH upregulates only HIF2α levels (51). In addition, it is worth mentioning that it has been observed that HIF1 and HIF2 have the opposite effects on regulation of proliferation in other cell types—while HIF2 promotes cell cycle progression, HIF1 inhibits it (52, 53). The participation of HIF2 in the regulation of proliferation by FSH was further confirmed by the demonstration that inhibitors of HIFs down-regulate bromo-deoxyuridine (BrdU) incorporation, cyclin D1 expression and c-Myc activity stimulated by FSH in immature Sertoli cells (51).

In summary, FSH positively regulates the proliferation of Sertoli cells by activating cAMP/PKA/ERK1/2 and PI3K/Akt/mTORC1 dependent-pathways, and by increasing the transcriptional activity of c-Myc and HIF2 and the expression of cyclin D1. The characterization of some of the signaling pathways regulated by FSH has been an important step toward the understanding of how this hormone promotes, on the one hand, Sertoli cell proliferation during fetal and early postnatal life and, on the other hand, maturation after cessation of mitosis at puberty. Nevertheless, many of the mechanisms by which FSH exerts its biological actions which, as mentioned above, vary with the developmental status of the animal, remain to be fully understood. Finally, yet importantly, the involvement of FSH-promoted autocrine factors in Sertoli cell proliferation should be considered.

Insulin Family of Growth Factors

The insulin family of growth factors—insulin, insulin like growth factors I (IGF-1) and II (IGF-2) and relaxin– are small

polypeptides that are responsible for the control of growth, metabolism, and reproductive functions. IGF-1 and IGF-2, which share 70% of their amino acid sequence, are ubiquitously expressed unlike insulin that is expressed in the pancreatic islets of Langerhans β-cells. The physiological effects of these peptides are mediated through the activation of two related tyrosine kinase receptors: the insulin receptor (InsR), and the IGF-1 receptor (IGF-1R). Ligand binding activates receptor tyrosine kinase activity and subsequently, phosphorylation of tyrosine residues in the receptor itself and a set of proteins known as insulin receptor substrates (IRS) occurs. Four IRS proteins that initiate intracellular pathways, named IRS1-4, have been identified (54). Activation of the receptor will lead to stimulation of two major signaling pathways: PI3K and ERK1/2, both of which are associated with proliferation, differentiation, metabolism, and cell survival.

The members of this family that have been more extensively studied in the context of testicular function are insulin and IGF-1. Utilizing different approaches, crucial roles for both peptides in testicular development were established. In their analysis of *Igf-1* knock-out (KO) mice, Baker et al. (55) showed the importance of IGF-1 for the development and fate of the mouse male gonad. Sertoli cell characteristics were not evaluated in these initial studies. Other studies, related specifically to Sertoli cell proliferation, indicated that IGF-1 effects take place both during embryonic and neonatal periods. It has been shown that Sertoli cells isolated from embryonic mouse testis express IGF-1 and IGF-1R and that IGF-1 treatment increases BrdU incorporation and promotes cell cycle progression (56). Furthermore, IGF-1 receptors were identified in neonatal Sertoli cells (57, 58) and a positive role of IGF-1 in regulating Sertoli cell proliferation in cultured cells from different species (rat, pig, and bull) was demonstrated (57, 59–61). Moreover, cultured Sertoli cells with inactivated IGF-1R showed decreased BrdU incorporation and cyclin D2 expression and increased p21Cip1 and p53 protein levels (62). As for the role of insulin, early studies showed that insulin exerts proliferative effects as well as IGF-1, however, as higher concentrations of insulin (micromolar) than those of IGF-1 (nanomolar) were required to elicit biological responses, it was suggested that insulin acted via IGF-1 receptors (63). More recently, additional support to the concept that both peptides, IGF-1 and insulin, are involved in proliferation of Sertoli cells was provided by analyzing mice lacking *Insr* and/or *Igf1r* specifically in Sertoli cells. Adult testes of mice lacking both *Insr* and *Igf1r* in Sertoli cells (SC-*Insr*;*Igf1r*) displayed a 72% reduction in testis size and a 79% reduction in daily sperm production. Reduced proliferation of immature Sertoli cells during late fetal and early neonatal development in these animals was also observed. However, despite the marked reduction in sperm production they were fertile indicating that the absence of IGF signaling in Sertoli cells does not impair spermatogenesis (64). As a whole, these investigations established that insulin and IGF-1 are essential components of the endocrine and paracrine network that regulates Sertoli cell proliferation.

Regarding the signal transduction pathways elicited by insulin/IGF-1 system in Sertoli cells, there is general agreement that they can activate PI3K/Akt and ERK1/2 signaling pathways and, additionally, that this is mediated by IRS2. With regard to the latter, it has been shown that adult *Irs2* KO mice show a 45% reduction in testis weight, with a reduction in the number of Sertoli cells, spermatogonia, spermatocytes, elongated spermatids, and spermatozoa, whereas testicular development in *Irs1* KO mice does not evince these reductions (65). A reduced testicular size was observed as early as the neonatal period, suggesting that testicular development impairment in *Irs2* KO mice also occurs in the fetal period. Altogether, these data indicate that IRS2, by mediating IGF-1 signaling during embryonic and early postnatal periods, plays a critical role in testicular development. The role of the ERK1/2 pathway in mediating IGF-1 effects on Sertoli cell proliferation had been suggested in experiments showing that the effects of IGF-1 on embryonic Sertoli cell proliferation were inhibited by a specific ERK1/2 pathway inhibitor (56). Regarding PI3K/Akt pathway, it has been shown that IGF-1 can stimulate this pathway in immature Sertoli cells (60). However, a direct relationship of the activation of PI3K/Akt pathway in mediating IGF-1 action in Sertoli cell proliferation has not been determined yet.

Interestingly, the insulin/IGF-1 signaling pathway has been proposed to play a role in mediating FSH effects in immature Sertoli cells. Initial studies on the regulation of IGF-1 system by FSH demonstrated that this gonadotropin stimulates IGF-1 and inhibits IGF binding protein (IGFBP) 3 secretion in Sertoli cells (66–69). By the fact that, in *Fshr* KO mice, testis weight, and Sertoli cell numbers were reduced ∼50% (70), while in SC-*Insr*;*Igf1r* KO mice, a greater reduction was observed (64), it has been suggested that the effects of FSH on the proliferation and differentiation of immature Sertoli cells are mediated, at least in part, by IGF-1. Pitetti et al. (64) studied the potential interaction between FSH and the insulin/IGF signaling pathway. The authors utilized an experimental model consisting of neonatal hemicastration of wild type and SC-*Insr*;*Igf1r* KO animals. While testis size and epididymal sperm counts were increased in hemicastrated wild type males, no effects were observed in hemicastrated SC-*Insr*;*Igf1r* mutant mice. Moreover, human recombinant FSH therapy did not increase testicular size and sperm output in SC-*Insr*;*Igf1r* KO mice. Based on these results the authors suggested that FSH requires the insulin/IGF-1 signaling pathway to mediate its proliferative effect on immature Sertoli cells. However, it has to be kept in mind that in these SC-*Insr*;*Igf1r* KO mice a reduction in FSHR and Akt signaling was also observed. Thus, the inability of Sertoli cells to proliferate after hemicastration or after FSH treatment might be accounted for by the reduction in both FSHR and Akt signaling. In summary, it can be concluded that the effects of FSH on immature Sertoli cells can be mediated, at least in part, by IGF-1 and that local production of IGF-1 is an important component of the intratesticular network involved in the regulation of Sertoli cell number, testis size and sperm output in mammals.

Relaxin is another member of the insulin-related peptide family involved in Sertoli cell proliferation. This peptide was first recognized for its important role during pregnancy and parturition (71, 72). Relaxin is structurally similar to insulin, but binds to GPCRs termed the relaxin family peptide receptors (RXFP 1 and 2) (73). Relaxin mRNA levels are higher in the

testis of immature rats than in the adult ones, which suggests an important role in an early period of life (74). Considering that relaxin and RXFP1 expression is found in immature Sertoli cells, an autocrine regulation has been predicted (75). Relaxin increases the incorporation of [^{3}H]-thymidine and the levels of proliferating cell nuclear antigen (PCNA) in Sertoli cell cultures. Relaxin-induced Sertoli cell proliferation involves activation of a Gi protein and activation of EKR1/2 and PI3K/Akt pathways (76). Supporting the hypothesis that relaxin has a role on Sertoli cell proliferation, it has been observed that KO mice for this gene have smaller testes (77). More recently, a crosstalk between FSH and relaxin at the end of the proliferative stage in rat Sertoli cells was demonstrated, and the authors postulate that whereas FSH action predominates and seems essential to direct cell maturation, relaxin seems to preferentially promote Sertoli cell proliferation (78, 79).

Activins and Inhibins

The gonadal peptides activins and inhibins belong to the transforming growth factor (TGF) β superfamily, and have important roles in reproduction and development. Activins and inhibins were discovered and named based on their abilities to stimulate or inhibit FSH release by gonadotrophs (80, 81). Activins are homodimers of two β inhibin subunits encoded by five genes designated *Inhba* to *Inhbe* (82–85). The most studied activins are those called activin A (βAβA) and activin B (βBβB). On the other hand, inhibins are heterodimers of one of the β subunits –βA or βB– with a common inhibin α-subunit, encoded by *Inha* gene, namely inhibin A (αβA), and inhibin B (αβB), respectively (82, 86).

All information related to activin regulation of Sertoli cell function has been obtained using activin A. Activin A is expressed in fetal and postnatal testis with variable cell localizations (87–91). In the fetal testis, Leydig cells are the main source of activin A in the mouse and human (92, 93). Barakat et al. (94) showed that Inh βA subunit is detected by immunohistochemistry in Sertoli cells, Leydig cells, peritubular myoid cells (PTMC), and different types of germ cells. This latter study, which included studies in newborn mice, also showed that testicular concentration of activin A is high during the period of Sertoli cell proliferation and then decreases to reach a low value that remains constant until adulthood. Considering that Buzzard et al. (89) had previously shown that neonatal PTMC in culture produce higher levels of activin A than Sertoli cells, it has been proposed that the high concentrations of activin A found in neonatal testis are due to activin A produced by PTMC.

Activin A signaling is mediated by binding to a type II receptor subunit, either ActRIIa or ActRIIb, which causes type II receptor to phosphorylate type I receptor, ActRIb (ALK4). Phosphorylated type I receptor recruits and phosphorylates SMAD2 and/or SMAD3, also called regulatory SMADs. The latter phosphorylated proteins dissociate from the receptor and oligomerize with the common SMAD4. This oligomer translocates to the nucleus and affects specific gene transcription. The relevance that activin A may have in Sertoli cell mitosis is pointed out by the presence of type II and type I receptors in mitotically active Sertoli cells (89, 95).

More than 20 years ago, at the time when the TGFβ peptide superfamily was being characterized, the need to investigate possible paracrine effects within the testis became apparent. To this respect, a synergistic effect of activin A with FSH on Sertoli cell proliferation in the neonatal period was described (89, 96). The effect of activin A on fetal Sertoli cell mitosis was analyzed in *Inhba* KO mice. *Inhba* KO males have significantly fewer Sertoli cells and a lower testis weight than wild type males at birth. Concomitantly, a reduction in BrdU- and PCNA-positive Sertoli cells and a decrease in cyclin D2 expression in Sertoli cells in these animals were observed (97). A study using genetic disruption of *Inhba* specifically in fetal Leydig cells (*Inhba* cKO) showed decreased proliferation of Sertoli cells, and the authors postulated that activin A produced by Leydig cells is the relevant paracrine regulator of fetal Sertoli cell mitosis (93). Noticeably, it has been shown that activin A is present in fetal human testis and that activin A increases Sertoli cell proliferation in fetal human testis in culture (92, 98). These results point out the physiological relevance of this peptide in fetal Sertoli cell mitosis.

The role of different activin receptors and of the SMAD signaling pathway in the proliferative effect of activin A has also been studied. It has been shown that mice with deletion of *ActrIIa* have smaller testis size and a reduced number of Sertoli cells (99). On the other hand, abrogation of ALK4/5/7 signaling in gonad cultures has been shown to promote a significant reduction in fetal Sertoli cell proliferation (100). As for the participation of SMAD signaling pathway, Itman et al. (101) showed that activin A promotes nuclear accumulation of SMAD3 rather than SMAD2 in proliferative Sertoli cell cultures. These results suggest that activin A signals preferentially through SMAD3 in immature Sertoli cells. Moreover, mice with conditional deletion of *Smad4* in Sertoli cells showed decreased Sertoli cell proliferation and a reduction in testis size (93). Altogether, these studies support the notion that activin receptors and SMAD3-SMAD4 signaling dependent pathways are involved in the regulation of Sertoli cell proliferation.

As mentioned before, activin A production decreases with the age of the animal. The most marked drop occurs at puberty at the time that Sertoli cells mature and are in a non-proliferative state (94). Even though activin A production decreases, it is present throughout adulthood, and investigators searched for a role of the peptide in maturing Sertoli cells. In this context, Nicholls et al. (102) using Sertoli cell cultures that have hallmarks of mature cells—tight junction formation, mitotic arrest and expression of maturity markers—showed that activin A inhibits tight junction formation between neighboring Sertoli cells. In addition, the authors showed that activin A induces proliferation of these mature Sertoli cells and increases cytokeratin 18 expression, a marker of immature Sertoli cells. These *in vitro* studies were complemented with an *in vivo* approach. Adult mice with increased systemic activin A levels showed a disruption of the BTB, an increase in the number of seminiferous tubules with severe spermatogenic defects and a decrease in testis weight. The authors concluded that the switch from high to low activin A levels during testis development is physiologically relevant and might be important for appropriate Sertoli cell function and fertility.

The role of activin B on Sertoli cell function is much less understood. Studies using *Inhbb* KO mice suggest that activin B is not involved in Sertoli cell proliferation or testicular development (103). However, further studies will be necessary to determine if activin B has any role in the regulation of Sertoli cell proliferation.

Regarding the inhibins, it has been shown that inhibin B is the major circulating inhibin in males and that it is produced mainly by Sertoli cells in the testis (92, 94, 104). Mice with a deletion of *Inha* have normal testicular development during embryogenesis but develop testicular tumors by 30 days of age. These tumors completely alter testicular architecture and render the mice infertile (105). Based on the latter observations, a marked inhibition of Sertoli cell proliferation by inhibin B was initially postulated. A few years later, it was shown that *Inha* KO mice, which do not produce inhibin B, have overproduction, and unbalanced action of activin A that might be responsible for the observed phenotype (106). In support of the latter hypothesis, it has been observed that the genetic deletion of *Smad3*, an important activin A signaling molecule, relieves the Sertoli cell tumor-forming phenotype of *Inha* KO mice (107, 108). Altogether, this evidence suggests that in adulthood inhibin B has no role by itself but plays a role in the modulation of activin A-induced Sertoli cell proliferation.

In summary, activin A stimulates Sertoli cell proliferation during fetal and postnatal period. Increasing levels of inhibin B at puberty may counteract activin A effects.

Cytokines

Cytokines are typically characterized as factors made by more than one cell type that act locally in an autocrine or paracrine fashion and play a pivotal role in the regulation of immune and non-immune cells. Different cell types in the testis under both physiological and pathological conditions produce cytokines (109, 110). Considering that the mammalian testis is a notable immune-privileged site, which protects haploid immunogenic germ cells from the harmful effects of immune responses, most studies focused on the effects of cytokines on the maintenance or disruption of the immune environment. However, a few addressed cytokine regulation of Sertoli cell proliferation.

Interleukin 1 (IL-1) exists as two major agonist isotypes, IL-1α, and IL-1β, and these cytokines have a naturally antagonist named IL-1 receptor antagonist (IL-1ra). In rodent testis, IL-1α, and IL-1β are produced by Sertoli cells, interstitial macrophages and Leydig cells (111–115). The production of IL-1α by human Sertoli and Leydig cells has also been demonstrated (116). Both IL-1s exert their effects by binding IL-1 receptor type I that is constitutively expressed in Sertoli cells (117, 118). As for Sertoli cell proliferation, it has been shown that IL-1α and IL-1β increase DNA synthesis and Sertoli cell number *in vitro* and that IL-1α has a more potent effect than IL-1β (118). Petersen et al. (119) explored the signaling pathways activated by IL-1α and their participation in the proliferative effects on Sertoli cells. The authors showed that IL-1α activates p38 MAPK and JNK pathways but not the ERK1/2 cascade in immature Sertoli cells, and that the p38 MAPK pathway mediates the mitogenic effect of IL-1α. IL-1α expression can be demonstrated at about 20 days of age increasing thereafter in the rat testis (120). Tumor necrosis factor (TNF) α is another cytokine with known effects in the pro-inflammatory and immunoregulatory responses, and apoptosis. TNFα is produced by interstitial macrophages, spermatocytes and spermatids in the adult testis (121). Only TNF receptor 1 has been detected in Sertoli cells and probably mediates TNFα biological actions (122). Petersen et al. (123) presented data consistent with a possible positive role of TNFα on Sertoli cell proliferation. Considering that neither IL-1α nor TNFα were detected in the immature testis, the possible role of these cytokines in Sertoli cell proliferation under physiological conditions is at least arguable.

In summary, the effects of cytokines on Sertoli cell proliferation under physiological conditions are unlikely, however, they may have some relevance under pathological conditions with elevated intratesticular cytokine levels.

MAIN FACTORS INVOLVED IN CESSATION OF PROLIFERATION AND IN MATURATION OF SERTOLI CELL

Cessation of proliferation of Sertoli cells is accompanied by a maturation process that consists in profound changes in gene expression, BTB establishment and the acquisition of full capacity to sustain developing germ cells. Therefore, the analysis of hormones and locally produced factors as well as the analysis of the signaling pathways and molecular mechanisms involved in cell cycle arrest and maturation of Sertoli cells are also relevant to the understanding of possible alterations in sperm production. Current knowledge of the role of androgens, estrogens, thyroid hormones, retinoic acid, and opioids in the cessation of proliferation and/or maturation will be summarized in the following sections.

Androgens

The role of androgens in male fertility and in the maintenance of spermatogenesis is well-known and has been extensively reviewed (124, 125). During embryogenesis, fetal Leydig cells secrete testosterone shortly after differentiation to ensure virilization of the male embryo, and this secretion gradually declines preceding birth (126). Another testosterone surge occurs following birth and the profile of this neonatal testosterone surge has been characterized in different species (127). Then, testosterone decreases to very low levels until the onset of puberty (128, 129). At puberty, serum testosterone rises again reaching adult levels that cause development of secondary sex characteristics and progressive acquisition of reproductive capacity.

Androgens exert most of their effects through genomic actions, which involve diffusion through the plasma membrane to bind the androgen receptor (AR), which is sequestered by heat shock proteins in the cytoplasm. The interaction of the steroid with the AR leads to a conformational change in AR which causes its release from heat shock proteins. Ligand-bound AR then translocates to the nucleus where it interacts with androgen response elements (AREs) in gene promoter regions, recruiting co-regulator proteins and regulating gene transcription. An

extensive review dealing with the mechanism by which AR acts as a ligand-dependent transcription factor was published by Heemers and Tindall (130).

AR is widely expressed in the rat testis, specifically in Sertoli, Leydig, and PTMC (131). The localization of AR in germ cells is controversial, with some studies showing absence of AR (131–134) and other studies presenting evidence of AR expression in germ cells (135–137). AR is absent in fetal rat Sertoli cells, and its expression becomes progressively stronger during postnatal development (131, 138, 139).

It was generally believed that androgens played little if any role in Sertoli cell proliferation in rodents, primarily because the AR is weakly expressed in Sertoli cells during early postnatal life. Precisely, the fact that the treatment of neonatal mice with testosterone propionate did not modify the expression of proliferation markers—c-Myc and PCNA—in immature Sertoli cells was interpreted as a consequence of the low levels of expression of AR (140). This hypothesis was questioned by studies in testicular feminized mice (Tfm, nonsense mutation in *Ar* gene resulting in a complete absence of nuclear receptor protein in all tissues) showing a decrease in the number of Sertoli cells in adulthood (141). However, a controversy arose on these observations as these animals suffer cryptorchidism that may be the cause of reduced Sertoli cell number. A year later, Atanassova et al. (142) also postulated a positive effect of androgens on Sertoli cell proliferation. These authors treated neonatal rats with the antiandrogen flutamide and observed reduced Sertoli cell number despite the presence of elevated FSH levels. The development of a Sertoli cell-selective *Ar* knockout mouse (SCARKO) opened up new possibilities for elucidating the role that androgens play in regulating Sertoli cell proliferation. In sharp contrast to what was observed in Tfm, flutamide-treated animals and ARKO—*Ar* KO mice with an analog phenotype to Tfm—models, the final number of Sertoli cells in SCARKO mice was unaltered (143). SCARKO animals conclusively evinced that AR expression in Sertoli cells is not required for attainment of a normal Sertoli cell number. Tan et al. (143) suggested that AR expression in other testicular cell types might be important for the modulatory role of testosterone on Sertoli cell proliferation. As PTMC express AR intensely throughout fetal and postnatal life, and, there is abundant evidence that PTMC secretions can modify Sertoli cell function (144, 145), it was hypothesized that androgens regulated Sertoli cell proliferation indirectly through their effects on PTMC. Interestingly, transgenic PTMC-$Ar^{-/y}$ mice exhibit decreased testicular weight and sperm count, and even though the authors did not determine the number of Sertoli cells, it is reasonable to think that the reduction in germ cell number in this model might be a consequence of a diminution in the number of Sertoli cells (146). Taking into account that there is evidence that activin A produced by PTMC can stimulate Sertoli cell proliferation (89), it was proposed that activin A might be the paracrine factor involved in the indirect action of androgens. The development of a transgenic model that prematurely expresses the AR specifically in Sertoli cells (TgSCAR) enabled to demonstrate that androgens, by acting specifically on Sertoli cell AR, induce cell maturation. Sertoli cell maturation in TgSCAR mice was demonstrated by the observation of accelerated postnatal formation of seminiferous tubular lumen and for elevated levels of mRNAs coding for tight junction or phagocytic function proteins. Additionally, a premature AR expression in Sertoli cells led to a reduction of the pool of immature cells available for FSH induced mitotic expansion and resulted in fewer Sertoli cells in adulthood (147). In agreement with these findings, Buzzard et al. (148) had previously shown that testosterone inhibited rat Sertoli cell proliferation in primary cultures through the induction of cell cycle inhibitors p21Cip1 and p27Kip1.

In addition to the classical AR-mediated androgen responses, the regulation by androgens of Sertoli cell function may involve non-classical responses (149, 150). Recently, a novel membrane receptor for androgens, which is unrelated to AR, was identified. The zinc transporter ZRT-and Irt-like Protein (ZIP) 9, one of the 14 members of the solute carrier family 39 (SLC39) that regulates zinc homeostasis, is now known to mediate some androgen actions (151). To this respect, the expression of ZIP9 and its participation in the regulation of claudin-1 and—5 expression and the assembly of tight junctions have been described in a Sertoli cell line (152). Thus, the contribution of ZIP9 to androgen regulation of Sertoli cell maturation should be considered.

In summary, while androgens were initially thought to have a positive effect on Sertoli cell proliferation, studies on transgenic mice suggest that androgen-dependent regulation of Sertoli cell proliferation is an indirect effect probably exerted through the secretion of a paracrine factor. Direct effects of androgens on Sertoli cells seem to be related to maturation of this cell type. The mechanisms participating in androgen regulation of Sertoli cell function remain to be fully elucidated.

Thyroid Hormones

Thyroid hormones T3 and T4 (TH) are critical regulators of growth, development, and metabolism in virtually all tissues. TH initiate biological responses via classical genomic pathways by binding to TH receptors (TRs) that are codified by two genes, *Tra,* and *Trb*. Alternative splicing of the RNA transcript of both genes generates several different protein isoforms. Only four of these TR isoforms, TRα1, TRβ1, TRβ2, and TRβ3, seem to bind TH and act as TRs (153, 154). Additionally, a 43-kDa truncated form of the nuclear receptor TRα1 (p43) synthesized by the use of an internal initiation site of translation in the TRα1 transcript was identified in the mitochondrial matrix (155). The p43 stimulates mitochondrial transcription and protein synthesis in the presence of T3 (156). Nongenomic effects of TH initiated by activation of a plasma membrane receptor integrin αvβ3 have also been described (157).

Testicular TR expression varies with the age of the animal, particularly in Sertoli cells. TRα1 is expressed in proliferating Sertoli cell nuclei, and its expression decreases coincident with the cessation of proliferation. On the other hand, TRβ1 mRNAs is expressed at low levels throughout lifespan and the corresponding protein is not detected (158). High-affinity binding sites for T3 have been observed in rat Sertoli cell mitochondria (159) and these results suggest the presence of the p43 receptor in this cell type. TH interactions with the integrin

αvβ3 receptor, which mediate rapid responses, have also been described in Sertoli cells (160).

Concerning the role of TH in immature Sertoli cells, an extensive body of data obtained from *in vivo* and *in vitro* models shows that TH inhibit Sertoli cell proliferation and stimulate their functional maturation in prepubertal testis through its interaction with different TH receptors. The central role of TH in regulating immature Sertoli cell function was highlighted by studying the effects of hypothyroidism and hyperthyroidism on neonatal rats. On the one hand, it was shown that early hypothyroidism causes an increase in testis size and in daily sperm production in adult rats (161), which is correlated with an increment in Sertoli cell number and with a delay in Sertoli cell maturation (162). On the other hand, high neonatal TH levels reduced the period of Sertoli cell proliferation, accelerated tubular lumen formation, and increased inhibin secretion (163). These results indicated that, additionally to its role in halting proliferation, TH also promote Sertoli cell maturation. A more recent study made similar observations on Sertoli cell proliferation and maturation as a consequence of neonatal hypothyroidism and hyperthyroidism, which were also induced by propylthiouracil (PTU) and T3 treatment, respectively. The total number of Sertoli cells per testis was significantly increased in PTU-treated mice in comparison to the controls, whereas the opposite occurred in T3-treated mice. Although the meiotic index and Sertoli cell spermatogenic efficiency were similar in all three experimental groups, the total daily sperm production per testis was significantly higher and lower than in control animals in PTU- and T3-treated mice, respectively (164).

Culture of immature Sertoli cells constitutes another experimental approach that was used to analyze the role of TH in Sertoli cell proliferation and maturation. In these *in vitro* studies, it was observed that TH inhibited FSH-stimulated Sertoli cell mitosis (165, 166) and that TH inhibition of Sertoli cell proliferation was accompanied by an increase in the expression of the cell cycle inhibitors p21Cip1 and p27Kip1 (148). It is worth mentioning that studies in KO animals for these cyclin-dependent kinase inhibitors (CDKIs) demonstrated that both are important inhibitors of Sertoli cell proliferation, and that loss of these CDKIs leads to a large increase in the adult Sertoli cell population, as well as an increase in daily sperm production and in testis weight (167). On the other hand, TH treatment stimulated the expression of Sertoli cell maturation markers such as inhibin B, clusterin, and AR and inhibited the expression of immature Sertoli cell markers such as aromatase and Anti-Müllerian hormone (AMH) (165, 168–171). Taking together, the evidence indicates that TH are central players in the transition from an immature to a functionally mature Sertoli cell phenotype.

Studies in KO animals for different THR (*Trα* KO and *Trβ* KO mice) were used to determine the roles of these receptors in mediating TH effects on Sertoli cells and testicular development (172). These studies on transgenic mice showed that TRα1, but not TRβ, is the receptor by which TH promote Sertoli cell maturation. This conclusion was further confirmed by analyzing a specific Sertoli cell *Trα1* mutant animals, $Tr\alpha^{AMI}$-SC (173). Additionally, it was shown that *p43* KO mice depict a testicular phenotype very similar to that observed in *Trα* KO and $Tr\alpha^{AMI}$-SC mice, suggesting that mitochondrial p43 receptor has a physiological role in Sertoli cell development (174).

Regarding the molecular mechanisms involved in the cessation of Sertoli cell proliferation by TH, the role of connexin 43 (Cx43), a constitutive protein of gap junctions that participates in the control of cell proliferation and tight junction formation, deserves special attention. To this respect, it was observed that the inhibitory effect of TH on Sertoli cell mitosis is associated with a time- and dose-dependent increase in Cx43 levels (175). The authors also showed that TH increased Cx43 levels in Sertoli cell cultures. Interestingly, specific Sertoli cell *Cx43* KO mice (SC-*Cx43*KO) showed sustained proliferation and delayed maturation of Sertoli cells in adulthood (176). Altogether, the results support the idea that Cx43, by promoting the tight junction formation between Sertoli cells, plays a pivotal role in the ability of TH to promote Sertoli cell maturation. The participation of p21Cip1 and p27Kip1 also merits consideration. Early studies showed that p27Kip1 levels in Sertoli cells are inversely related to the proliferative activity of these cells (177). Additionally, it has been shown that TH status affects p27Kip1 expression in neonatal Sertoli cells *in vivo* (178). It is well-known that p27Kip1 and to a lesser extent p21Cip1 are primarily regulated through changes in proteolytic degradation (179, 180), however, conclusive evidence for the relevance of this mechanism in Sertoli cell physiology has not been obtained yet. Other molecular mechanisms underlying the ability of TH to promote Sertoli cell cycle arrest are those that involve transcription factors c-Myc and JunD, activator and repressor, respectively of cyclin-dependent kinase 4 (CDK4) expression. By utilizing $Tr\alpha^{AMI}$-SC and *p43* KO animals, up-regulation of CDK4 and c-Myc was observed and it was postulated that these proteins are main factors controlling the proliferation of Sertoli cells (173, 174). However, direct effects of TH on c-Myc expression or on transcriptional activity in Sertoli cells remain to be determined.

The participation of signal transduction pathways in TH effects on immature Sertoli cells was scarcely analyzed. To this respect, Sun et al. (181) postulated that the effects of TH on Sertoli cell proliferation are dependent on inhibition of PI3K/Akt signaling and that this effect is mediated by the cell membrane receptor integrin αvβ3.

Finally yet importantly, given that one of the well-recognized functions of TH is related to energetic metabolism that is tightly associated with cell cycle progression, it can be proposed that the effect of TH on Sertoli cell proliferation may be linked to metabolic regulation. In this context, it might be hypothesized that TH utilize cellular energetic sensors such as AMPK and Sirtuins as mediators of their actions on immature Sertoli cells.

AMPK functions as a key energy-sensing kinase by virtue of its exquisite sensitivity to the cellular AMP/ATP ratio. An increase in the latter ratio promotes AMPK phosphorylation and activation by upstream kinases. Recently, the role of AMPK in cell growth and proliferation has captured attention. It has been demonstrated that AMPK activation causes G1/S phase cell cycle arrest in several cell lines (182, 183) and also that mTORC1 signaling can be downregulated by AMPK (184). Riera

et al. (45) have shown that AMPK activation reduces FSH-stimulated Sertoli cell proliferation. Activation of AMPK in FSH-stimulated conditions increases p19INK4d, p27Kip1, and p21Cip1 expression. As mentioned before, the regulation of p21Cip1 and p27Kip1 protein levels in response to TH has previously been related to the cessation of proliferation in FSH-stimulated Sertoli cells (148). Noticeably, TH activate AMPK in several cell lines (185–187). Altogether, the above-mentioned results let us speculate that AMPK activation may be involved in the mechanism of action of TH to regulate the transition of Sertoli cells from the mitotic to the postmitotic state during early postnatal development.

Sirtuins are metabolic sensors that have been implicated in a wide range of cellular processes. The mammalian Sir2 family consists of seven members (SIRT1-7) of NAD^+ dependent type III histone and protein deacetylases. The most extensive studies of these enzymes were conducted toward functions of SIRT1, which is the founding member of this family. Beyond histone deacetylation, this enzyme also deacetylates many non-histone proteins that are involved in several processes ranging from cell cycle regulation to energy homeostasis. Noteworthy, *Sirt1* KO animals are infertile showing decreased testis size and sperm quality, and this fact is accompanied by Sertoli cell immaturity (188). As a consequence, a physiological relevance of SIRT1 in Sertoli cell maturation has been proposed. It has been shown that SIRT1 is present in immature Sertoli cells at least up to 30-day-old rats and that SIRT1 expression in Sertoli cells decreases with the age of the animal. Additionally, SIRT1 activation markedly decreases proliferation and antagonizes FSH action in immature Sertoli cells. The molecular mechanisms involved in antiproliferative effects of SIRT1 activation were also studied. Activation of SIRT1 decreased cyclin D1 and D2 levels and increased p21Cip1 mRNA levels. SIRT1 activation also decreased c-Myc transcriptional activity (189). Remarkably, TH regulate SIRT1 expression and activity in different experimental models (190–192). SIRT1 activation might also be a molecular mechanism utilized by TH operating in immature Sertoli cells at the time of cessation of proliferation and terminal maturation of this cell type aimed to sustain spermatogenesis. However, experimental evidence for the involvement of AMPK and SIRT1 in the mechanism of action utilized by TH to regulate Sertoli cell proliferation has not been obtained yet.

In summary, studies performed so far have demonstrated that TH have a central role in the cessation of Sertoli cell proliferation and promote Sertoli cell maturation through TRα1 and p43 receptors. The mechanisms participating in these processes involve the regulation of Cx43, c-Myc, p21Cip1, and p27Kip1.

Estrogens

Estrogens play important roles in the regulation of testis development and spermatogenesis (193, 194). 17β-estradiol (E2) is the pre-dominant and most active estrogen produced from testosterone by aromatase enzyme cytochrome P45019 A1, encoded by the *Cyp19a1* gene (195). In males, E2 is present in low concentrations in blood, but its concentration in semen and the rete testis can reach values even higher than in female serum, suggesting a role for estrogens within the testis (196). Sertoli and Leydig cells, spermatocytes and spermatids express the aromatase enzyme (197, 198). Sertoli cells are the major source of estrogens in immature rats whereas Leydig cells are the main source in adult animals (197, 199). Sertoli cell E2 production is regulated by FSH through the increase of Cyp19a1 expression (200). This response of the Sertoli cells to FSH in terms of aromatase activity and E2 secretion markedly declines with age (201).

Genomic actions of estrogens are mediated by the classical nuclear estrogen receptor alpha (ERα or ESR1) and estrogen receptor beta (ERβ or ESR2). In addition to the genomic actions, rapid signaling events have been described. These latter rapid effects may be mediated by: (a) ERα and ERβ localized at or near the plasma membrane (202), (b) truncated variants of ERα called ERα-46 or ERα-36 (203, 204), and/or (c) G protein-coupled estrogen receptor (GPER or GPR30) (205). The rapid responses include activation of different downstream signaling pathways. It has been suggested that receptor post-translational lipid modifications, such as palmitoylation, can play a role facilitating membrane localization of ER (206).

Regarding ER expression in the testis, *in situ* hybridization and immnohistochemical studies carried out in rats at all ages suggested that ERβ was present in nuclei of Sertoli and Leydig cells, whereas ERα was only present in the interstitial space (207, 208). Years later, in studies performed in rat Sertoli cell cultures and utilizing more sensitive techniques, the presence of ERα was demonstrated (209, 210). Lucas et al. (210) found that ERα protein levels decrease, whereas ERβ protein levels increase in Sertoli cells with the age of the animals. There existed conflicting data regarding GPER expression in human and rodent testis (211–215). A careful study confirmed the presence of GPER in Sertoli cells from immature rats (216). Furthermore, GPER was immunodetected in the endoplasmic reticulum and Golgi apparatus, whereas almost no localization in the plasma membrane was observed (217).

One of the first approaches employed to assess the effect of estrogens on Sertoli cell proliferation consisted of the *in vivo* administration of estrogens to rats. In this regard, estrogen treatment reduced Sertoli cell number when administered during proliferative periods (218). Similarly, results obtained in studies carried out *in vivo* using an aromatase inhibitor and a non-specific ER antagonist (ICI 182,780) suggested that Sertoli cell proliferation diminishes by activation of estrogen receptors (219, 220). As ICI 182,780 is able to antagonize the effect of both ERα and ERβ, the results obtained do not distinguish the type of receptor participating in the observed biological effect.

In order to clarify the role of estrogens on testis development, several models of transgenic mice were studied. Among them are those mice overexpressing aromatase (221), *Cyp19a1* KO (222), *Erα* KO (223–225), and *Erβ* KO (225, 226). These transgenic mice were not selective for Sertoli cells, and due to the pleiotropic actions of estrogens, the phenotypes observed could not be straightforwardly attributed to direct actions of estrogens on Sertoli cell proliferation.

Studies performed in isolated Sertoli cells using specific agonists and antagonists of ERα and ERβ shed some light to the role of estrogens in proliferation. Estrogens might regulate both proliferation and cell maturation depending on the ER

isoform through which they exert the effect. While estrogens modulate Sertoli cell proliferation through ERα, cell cycle exit, and differentiation involve ERβ. Taking into account that ERα expression decreases while ERβ expression increases with the age of the animals, it was postulated that the ERα/ERβ ratio is physiologically relevant to determine the end of cell proliferation and the start of cell differentiation (210).

Concerning the signaling pathways involved in estrogen action in Sertoli cells, Lucas et al. (210) have shown that the interaction of E2 with ERα promotes cell proliferation through the activation of NFκB in a PI3K- and a ERK1/2-dependent manner and that this is accompanied by cyclin D1 induction. On the other hand, the interaction of E2 with ERβ promotes cell cycle exit and cell maturation through the activation of CREB in a PI3K-dependent manner and this leads to the expression of the Sertoli cell differentiation markers –p27Kip, GATA1, and DMRT1. More recent studies have shown that not only are the classical receptors involved in Sertoli cell proliferation but GPER as well. To this respect, Yang et al. (227) have shown that GPER triggers the activation of Src/PI3K/Akt pathway which is involved in E2-induced Sertoli cell proliferation via regulating the expression of S-phase kinase-associated protein 2 (Skp2). Additional studies destined to evaluate the participation of GPER, either alone or in conjunction with ERs, will be necessary for our overall understanding of estrogen biological function in Sertoli cells.

In summary, investigations performed so far have led to the conclusion that estrogens increase proliferation of Sertoli cells through ERα and GPER. On the other hand, at the end of the proliferative period estrogens promote cessation of proliferation and cell maturation through ERβ.

Retinoic Acid

It has been recognized for decades that signaling through vitamin A is essential for male reproduction. The biologically active form of vitamin A is retinoic acid (RA), which includes all-trans-RA (atRA), and 9-cis-RA (9-cRA). atRA is synthesized and also stored in lipid droplets in the testis (228–231). atRA content of the testis is practically independent from the plasma levels of this metabolite (232), suggesting that endogenous production of atRA has a vital importance for the maintenance of atRA-dependent processes in the seminiferous tubules. Cavazzini et al. (228) showed that atRA synthesizing activity rises about 5-fold at the time of transition to the non-proliferative phenotype of Sertoli cells and that it continues nearly constant thereafter. On the other hand, Raverdeaua et al. (233) showed that preleptotene spermatocytes may be another source of atRA at the time of meiotic initiation. It is worth mentioning that RA may have biological roles in all testicular cell types including germ cells at different stages of maturation. To this respect, several studies demonstrated that vitamin A deficiency in rats induces a progressive loss of germ cells, ultimately yielding seminiferous tubules that contain only Sertoli cells and premeiotic germ cells (234–237).

The actions of RA are mediated through specific nuclear receptors, the so-called retinoic acid receptors (RAR), which work as ligand-dependent transcription factors and that form heterodimers with retinoic X receptors (RXR). There exist three major subtypes of both, RAR protein (α, β, and γ) and RXR protein (α, β, and γ). Expression of various subtypes of RAR and RXR in Sertoli cells of fetal, neonatal, and adult animals has been demonstrated. Particularly, it has been observed that mitotically active rodent Sertoli cells express RARα and β and RXRα and γ (230, 238–240).

The regulation of Sertoli cell mitosis by atRA was evaluated in *in vitro* studies. Buzzard et al. (148) demonstrated that atRA decreases FSH-stimulated [^{3}H]-thymidine and BrdU incorporation in cultures of immature Sertoli cells. Concomitantly, atRA increases p21Cip1 and p27Kip1 expression, proteins that are widely known to be involved in cell cycle arrest and in differentiation of Sertoli cells. Additionally, Nicholls et al. (241) showed that atRA inhibits activin A-stimulated Sertoli cell proliferation. Furthermore, the authors showed that inhibition of cell proliferation is accompanied by a decrease in activin A-stimulated cyclin E1 expression and by an increase in the levels of the cell cycle inhibitor p15INK4. These *in vitro* studies suggest that atRA participates in cessation of Sertoli cell proliferation. As previously mentioned, the cessation of Sertoli cell mitosis is accompanied by the formation of inter-Sertoli cell tight junctions, the main component of the BTB in seminiferous tubules. Noticeably, Nicholls et al. (241) also showed that atRA increases transepithelial electrical resistance (TER), a measurement of tight junctions integrity in Sertoli cell cultures, and promotes plasma membrane localization of the tight junction-related proteins claudin-11 and Tjp1. Altogether, these observations are consistent with an anti-proliferative and pro-differentiative role of RA.

The role of RARs and RXRs proteins in testicular physiology has also been analyzed by utilizing total or selective KO animals. *Rarα*, *Rarγ*, or *Rxrβ* null animals have abnormal testicular histology and are infertile, whereas *Rarβ* and *Rxrγ* null males are fertile, and their testes and genital tract are histologically normal throughout life (242–247). *Rxrα* null fetuses died *in utero*, thus, its precise role in testicular function could not be defined (248). All these previous studies focused on the germ cell population while no specific attention to possible changes in Sertoli cell physiology was paid. Analyzing selective KO animals, Vernet et al. (249) proposed a possible role of RARα in the regulation of Sertoli cell maturation. The latter authors showed in Sertoli cell-specific *Rarα*-conditional KO mice that there is a marked impairment of Sertoli cell capacity to support germ cell development (249). In addition, Hasegawa and Saga (250) evaluated the impact of the overexpression of a dominant-negative form of the RARα receptor (dn-RARα) in Sertoli cells. They observed that the BTB was disrupted during specific seminiferous tubule stages and postulated that this is partially due to a reduction in occludin expression. A role of RA in BTB function is further supported by the observation of partial disruption of tight junctions in vitamin A deficient rats and in *Rarα* KO mice (251–253).

In summary, results obtained so far are consistent with a role of RA in cessation of proliferation and in maturation of Sertoli cells.

Opioids

Opioids are also present in the male gonad and are involved in the local control of testicular function. Opioids, such as proopiomelanocortin (POMC), α-melanocyte-stimulating hormone (αMSH), and β-endorphin, are mainly produced in Leydig cells and exert direct paracrine actions on Sertoli cells (254–256). Additionally, high affinity opioid binding sites in Sertoli cells obtained from immature and adult rats have been described (257). Later on three major classes of opioid receptors—mu, delta, and kappa—in Sertoli cells were described (258). As for Sertoli cell proliferation, few studies have focused on this issue. Orth (259), utilizing the opioid receptor blocker naloxone, showed that endogenous opiate-like peptides inhibit the proliferative effects of FSH in fetal rat testis. Moreover, the same research group demonstrated that endorphin suppresses FSH-stimulated proliferation of isolated neonatal Sertoli cells possibly through activation of Gi (260). Sixteen years later, da Silva et al. (261) showed that neonatal treatment with naloxone increases the number of Sertoli cells and daily sperm production per testis in adult animals.

Results available so far are consistent with a negative effect of opioids on FSH-stimulated Sertoli cell proliferation.

PHARMACOLOGICAL AGENTS AND XENOBIOTICS MAY AFFECT SERTOLI CELL PROLIFERATION AND MATURATION

Epidemiological, clinical, and experimental studies suggest that there are multiple possible causes of the progressive decrease in male reproductive function observed over the past 50 years. Drugs used for the treatment of several pathologies or exposure to xenobiotics in early stages of life may alter testicular function and condition future fertility. Current knowledge of the effects of pharmacological agents and xenobiotics on Sertoli cell proliferation and maturation will be summarized in the following sections.

Pharmacological Agents

Chemotherapy drugs are the most extensively studied in terms of their possible testicular toxicity. It is known that male fertility is affected by chemotherapy treatment. For example, men treated with alkylating agents show oligospermia or azoospermia as well as alterations in the histology of the testis (262–264). Although the deleterious effects caused by chemotherapy in adults are well-known, mainly attributed to their cytotoxic action on germ cells, little is known about possible effects on the Sertoli cell population. Few studies have addressed the possible gonadotoxic effect of chemotherapeutic agents during childhood and puberty. In fact, it was postulated that chemotherapeutic drugs were less harmful when used in the prepubertal stage because the testis was considered quiescent (265, 266). However, studies with cohorts of patients who received high doses of chemotherapy before puberty clearly show an increased risk of infertility (267, 268). Testis damage might be related to the loss of germ cell population and/or alterations in the Sertoli cell proliferation and maturation processes that take place in the immature testis. The latter hypothesis is under debate and further studies will be necessary to definitively sustain that chemotherapy drugs affect Sertoli cell proliferation. Thirty years ago, it was observed that *in vivo* intratesticular injection of cytosine arabinoside, a chemotherapeutic agent, inhibited Sertoli cell proliferation (4). Additionally, it has been observed that treatment of neonatal rats with a single dose of doxorubicin, beyond promoting apoptosis in germ line stem cells, reduced the rate of increase of Sertoli cells leading to a decrease in the final number of them (269). Tremblay and Delbes (270) have also recently observed that doxorubicin decreases Sertoli cell number in culture. In contrast, Nurmio et al. (271) proposed that the germ cell population is the target of doxorubicin toxicity. As for other drugs, it has been shown in immature Sertoli cells in culture that acrolein—a metabolite of cyclophosphamide—induces cytoskeletal changes and oxidative stress (272). On the other hand, a marked loss of germ cells and no change in the Sertoli cell number were observed in neonatal mice testis treated *in vitro* with cisplatin, doxorubicin or the active metabolite of cyclophosphamide—phosphoramide mustard (273).

Some nucleoside analogs, such as acyclovir and ganciclovir, are used as antiviral agents to treat infections caused by herpes simplex virus, cytomegalovirus, varicella zoster virus, and other viruses. Studies performed in adult animals showed that ganciclovir or acyclovir treatment decreases testis weight and sperm count and increases the number of spermatozoa with abnormalities in head and tail (274, 275). On the other hand, Nihi et al. (276) showed that treatment of pregnant mice with a high dose of ganciclovir decreases the number of gonocytes in fetal testis. In addition, these authors showed that animals that have been exposed *in utero* to ganciclovir present decreased adult testis weight concomitant with an increase in the number of seminiferous tubules with partial or complete absence of germ cells. However, the authors did not observe differences in Sertoli cell number per tubular cross section and suggested that the effects induced *in utero* by ganciclovir may result from direct effects on developing germ cells and/or may be secondary to the dysfunction of Sertoli cells. In support of the latter assumption, Qiu et al. (277) showed in the Sertoli cell line SerW3 that ganciclovir and acyclovir induce a decrease in Cx43 expression, a protein that is essential for proper sertoli cell maturation.

Nonsteroidal antiinflammatory drugs and analgesic drugs, such as ibuprofen—isobutylphenylpropionic acid—and paracetamol—acetyl-p-aminophenol–, are widely used to treat inflammation and pain and commonly prescribed in pregnant women and in children. Just a couple of studies analyzed the effect of these drugs in the Sertoli cell population. Ben Maamar et al. (278) showed that *in vitro* exposure of fetal human testis to ibuprofen does not modify the number of Sertoli cells but decreases AMH and SOX9 expression, suggesting a role in Sertoli cell maturation. Recently, Rossitto et al. (279) showed that treatment of pregnant mice with a combination of paracetamol and ibuprofen promotes a reduction of fetal germ cells mitosis and a decrease in sperm count in offspring's adulthood. These authors also showed that this treatment does not modify fetal or postnatal Sertoli cell proliferation but induces a delay in Sertoli cell maturation. Altogether, these

studies support the idea that analgesic drugs may alter Sertoli cell maturation.

Metformin—dimethylbiguanide—is one of the most widely used anti-hyperglycemic agent for treating adult patients with type 2 diabetes. Nowadays, its role as a therapeutic agent is expanding and metformin constitutes the treatment of choice in cases of pregnancy disorders, such as gestational diabetes mellitus or preeclampsia, and also in polycystic ovarian syndrome (280, 281). Considering that metformin can cross the placental barrier, fetuses are exposed to the drug (282). In children, the incidence of both type 2 diabetes and obesity has risen at staggering rates and metformin has started to be used in the pediatric population (283–285). Numerous studies have demonstrated that in addition to its strong antidiabetic properties, metformin shows an anti-proliferative activity in cancer cells (286–288). Despite the latter findings, few studies have analyzed the effect of this drug in non-cancer cells. Particularly in the testis, Tartarin et al. (289) have shown that *in vivo* administration of metformin to pregnant mice reduces the number of Sertoli cells in fetal life and at birth in male offspring. On the other hand, Faure et al. (290) showed that metformin inhibits Sertoli cell proliferation and increases p21Cip1 levels *in vitro* and that treatment of chickens for 3 weeks decreases testis weight and seminiferous tubules diameter. More recently, Rindone et al. (291) showed that metformin decreases FSH-stimulated neonatal rat Sertoli cell proliferation *in vitro*. Concomitantly, a reduction in FSH-stimulated cyclins D1 and D2 and an increase in p21Cip1 expression were observed as a result of metformin treatment. Altogether, these studies support the notion that metformin decreases fetal and postnatal Sertoli cell proliferation.

The mechanism of action through which metformin exerts its effects has not been completely elucidated. On the one hand, it has been shown that in the liver, metformin is able to inhibit the mitochondrial isoform of the enzyme glycerol phosphate dehydrogenase, resulting in a decrease in the levels of dihydroxyacetone phosphate and an increase in the NADH/NAD^+ ratio in the cytoplasm (292). On the other hand, it has been shown that metformin partially inhibits the complex I of the respiratory chain, which produces a decrease in cellular energy levels that results in the activation of the AMPK (293, 294). As mentioned before, it has been demonstrated that AMPK activation inhibits Sertoli cell proliferation (45). In this context, it has been shown that metformin activates AMPK in mitotically active Sertoli cells (290, 291). In addition, Rindone et al. (291) showed that metformin inhibits FSH-stimulated mTORC1/p70S6K pathway, a signaling pathway that is involved in FSH-stimulation of Sertoli cell proliferation (45). These studies suggest that metformin can counteract the effects of FSH on Sertoli cell proliferation by modulating AMPK and mTORC1/p70S6K pathways. Bearing in mind that metformin is now being used in pregnant women, potentially gaining access to male fetuses, and in children at the same time as Sertoli cells proliferate, attention should be paid to a possible alteration in the final number of Sertoli cells in adulthood.

In summary, experimental evidence for chemotherapy, antiviral, analgesic, and anti-hyperglycemic drugs suggests that they may potentially affect the final number and/or the maturation of Sertoli cells and consequently sperm counts in adulthood. Longitudinal studies evaluating fertility in patients treated for prolonged periods with the above-mentioned drugs would be helpful to gain full confidence in their use at any stage of their lifespan.

Xenobiotics

Exposure to environmental foreign chemical substances derived from modern life style represents a growing concern due to the impact of these pollutants on developmental and reproductive functions in mammals. Chemical compounds that derive from industrial manufacturing, pesticides and herbicides utilized in agricultural practices, waste accumulation, and burning residues, generally termed xenobiotics, are only a few examples of the contaminants that affect human daily life. Nowadays, it is well-known that testis function is a primary target of a large number of pollutants. Delayed establishment of spermatogenesis (295), impaired differentiation of internal and external male genital structures (296), reduced sperm production and disruption of the hypothalamic–pituitary–gonadal axis (297), decreased anogenital distance and decreased prostate and seminal vesicle weights (298, 299), among others, represent just some of the multiple observations performed.

Several studies have focused on the understanding of the mechanisms involved in the damage of the male reproductive system. Most of the xenobiotics act as endocrine disruptors in multiple organs by mimicking naturally occurring hormones or by binding to hormone receptors (e.g., ER, AR, TR, and so forth) and blocking the action of the hormone. In some cases, xenobiotics induce oxidative stress, decrease activities of antioxidant enzymes and produce perturbation of the tight junctional proteins (300, 301). In the testis, xenobiotics can alter BTB integrity by promoting a loss of gap junction function (302). Environmental toxicants also induce testicular cell apoptosis, and the role of the Fas/FasL signaling pathway has been demonstrated (303, 304). The latter reports represent a brief list of the numerous effects of xenobiotics that have been observed on testicular function. The majority of the studies were performed in adult animals, while there are few investigations on the possible role of xenobiotics in early periods of life when Sertoli cells are proliferating. In the next paragraphs, available information on the effects of toxicants on Sertoli cells during early periods of life is presented.

Phthalic acid esters are widespread in the environment. These compounds are used as plasticizers in food packaging, some children's products and some polyvinyl chloride (PVC) medical devices. *In vitro* experiments show that low levels of mono (2-ethylhexyl) phthalate (MEHP) disrupt Sertoli cell-gonocyte physical interaction and suppress Sertoli cell proliferation (305). In addition, treatment of neonatal rats with low levels of di (2-ethylhexyl) phthalate (DEHP) and its metabolite, MEHP, induces a decrease in Sertoli cell proliferation accompanied by a decrease in cyclin D2 levels (306). More recently, it has been shown that MEHP can disrupt prepubertal Sertoli cell proliferation by increasing intracellular ROS levels (307). On the other hand, it has been shown that another plasticizer of the phthalate family, mono-n-butyl phthalate (MBP), induces immature Sertoli cell proliferation up-regulating ERK1/2 signaling pathway (308).

Bisphenol A (BPA) is a monomer used in the manufacture of polycarbonate plastics and epoxy resins that are present in multitude of consumer products. BPA is an estrogenic endocrine disruptor very well-known for its ubiquitous presence and its effects on male reproduction (309). It has been shown that BPA alters Sertoli cell proliferation positively or negatively in a highly dose dependent manner (310). Additional compounds that can act as endocrine disruptors have been analyzed. It has been shown that oral exposure to the pesticide methoxychlor, a compound with estrogenic/antiandrogenic effects, reduces the number of Sertoli cells. Consistent with the importance of Sertoli cell number on daily sperm production, the study also shows that adult testicular weight and the amount of epididymal spermatozoa are significantly reduced (311). Zearalenone, a mycotoxin that is present in human and animal food and that has estrogenic activity, produces Sertoli cell cycle arrest that is mediated by alterations in PI3K/Akt/mTORC1 signaling (312).

Polychlorinated biphenyls, commonly called PCBs, are mixtures of chlorinated compounds that were used as insulation, coolants, and lubricants in transformers, capacitors, and other electrical equipment. They were also used in plasticizers, surface coatings, inks, adhesives, pesticides, and other products. PCBs cause hypothyroidism in animals (313). Regarding Sertoli cell proliferation, continuous exposure of lactating female rats to PCBs increases testis weight, sperm production, and Sertoli cell number in the adult male offspring. The observed increase in Sertoli cell number has been directly related to the hypothyroidism that these animals present (314, 315).

Our research group is presently investigating a possible deleterious effect of glyphosate, a widely used herbicide in agriculture, on Sertoli cell function. We have observed that glyphosate and particularly its commercial formulation Roundup decreases FSH-stimulated BrdU incorporation in Sertoli cell cultures. A decrease in cyclins and an increase in cell cycle

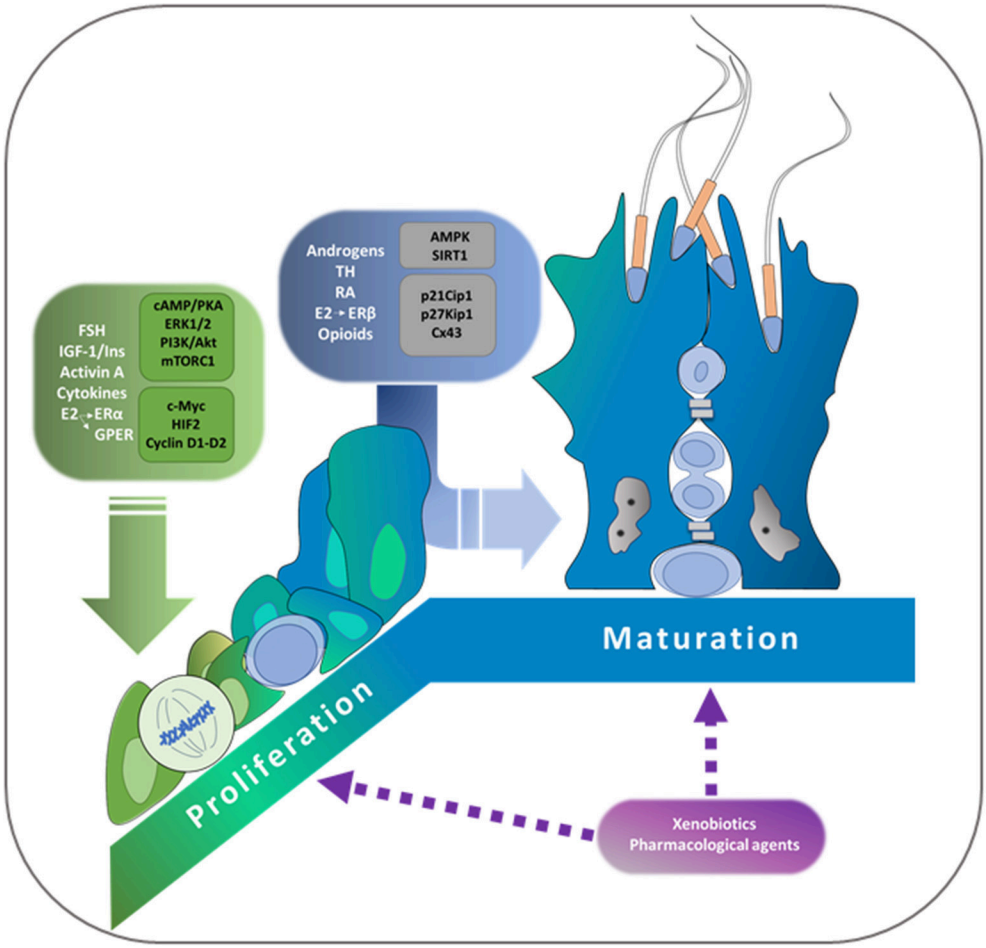

FIGURE 1 | Schematic representation of the main regulators of proliferation and of cessation of proliferation and/or maturation of Sertoli cells. Hormones and paracrine factors that stimulate proliferation are depicted in green and those that promote the cessation of proliferation and/or maturation are depicted in blue. Signal transduction pathways and possible mechanisms involved are also included in the schema. FSH, Follicle-Stimulating Hormone; E2, 17β-estradiol; ERα, Estrogen Receptor α; GPER, G protein-coupled estrogen receptor; IGF-1, Insulin-like growth factor 1; ins, insulin; PI3K, phosphatidyl-inositide-3 kinase; ERK1/2, extracellular regulated kinase 1/2; PKA, cAMP-dependent kinase; mTORC1, mammalian target of rapamycin complex 1; HIF2, Hypoxia inducible factor 2; TH, thyroid hormones; RA, retinoic acid; ERβ, Estrogen Receptor β; AMPK, AMP-activated protein kinase; SIRT1, sirtuin 1; Cx43, connexin 43.

inhibitors expression were also observed, and these results suggest that Roundup may affect Sertoli cell proliferation. *In vivo* studies are currently being performed in order to establish the actual impact of this herbicide in early periods of life.

Finally, gap junctions are potential targets for many environmental compounds. At the gonadal level, by using the Sertoli cell line SerW3, it has been reported that gap junctions and Cx43 expression are particularly altered in response to a large number of xenobiotics, such as toxins, xenoestrogens, pesticides, herbicides, heavy metals, and non-ylphenol (316, 317). The compelling evidence showing that Cx43 is essential for Sertoli and germ cell proliferation, differentiation, and survival and that environmental toxicants can interfere with normal Sertoli cell proliferation by altering Cx43 expression has been extensively reviewed by Pointis et al. (318).

In summary, experimental evidence indicate that Sertoli cell proliferation can also be a process that may be disturbed as a result of the exposure to environmental toxicants.

CONCLUSION

The final number of Sertoli cells reached during the proliferative periods determines both adult testicular size and sperm production capacity in adulthood. This final number of Sertoli cells results from events in fetal, neonatal and peripubertal life. Additionally, terminal differentiation of Sertoli cells, which involves loss of proliferative activity, formation of inter-Sertoli cell tight junctions and establishment of the BTB, is necessary to sustain spermatogenesis. Thus, a perfectly synchronized orchestra involving hormones, signal transduction pathways and molecular mechanisms that play to control Sertoli cell proliferation, and to promote the acquisition of a mature Sertoli cell phenotype is determinant for the future fertility. We have tried to summarize what is known about molecular mechanisms controlling Sertoli cell proliferation and maturation. A few examples of how exposure to environmental toxicants or pharmacological agents in early periods of life can compromise Sertoli cell number are discussed. The topics reviewed are summarized in **Figure 1**.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Berndtson WE, Igboeli G, Pickett BW. Relationship of absolute numbers of Sertoli cells to testicular size and spermatogenesis in young beef bulls. *J Anim Sci.* (1987) 64:241–6. doi: 10.2527/jas1987.641241x
2. Johnson L, Zane RS, Petty CS, Neaves WB. Quantification of the human Sertoli cell population: its distribution, relation to germ cell numbers, and age-related decline. *Biol Reprod.* (1984) 31:785–95. doi: 10.1095/biolreprod31.4.785
3. Griswold MD. Interactions between germ cells and Sertoli cells in the testis. *Biol Reprod.* (1995) 52:211–6. doi: 10.1095/biolreprod52.2.211
4. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology.* (1988) 122:787–94. doi: 10.1210/endo-122-3-787
5. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction.* (2003) 125:769–84. doi: 10.1530/rep.0.1250769
6. Sertoli E. Dell'esistenza di particolari cellule ramificate nei canalicoli seminiferi del testiculo umano. *Il Morgagni.* (1865) 7:31–9.
7. Parvinen M. Regulation of the seminiferous epithelium. *Endocr Rev.* (1982) 3:404–17. doi: 10.1210/edrv-3-4-404
8. Russell LD, Griswold MD. *The Sertoli Cell*. Clearwater, FL: Cache River Press (1993).
9. Skinner MK, Griswold MD. *Sertoli Cell Biology*. Amsterdam: Elsevier (2005).
10. Clermont Y, Perey B. Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am J Anatomy.* (1957) 100:241–67. doi: 10.1002/aja.1001000205
11. Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis *in vivo* and in organ culture. *Biol Reprod.* (1971) 4:84–7. doi: 10.1093/biolreprod/4.1.84
12. Griswold MD, Solari A, Tung PS, Fritz IB. Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. *Mol Cell Endocrinol.* (1977) 7:151–65. doi: 10.1016/0303-7207(77)90064-8
13. Orth JM. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat Rec.* (1982) 203:485–92. doi: 10.1002/ar.1092030408
14. Orth JM. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. *Endocrinology.* (1984) 115:1248–55. doi: 10.1210/endo-115-4-1248
15. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *BMJ.* (1992) 305:609–13. doi: 10.1136/bmj.305.6854.609
16. Aitken RJ. Human spermatozoa: revelations on the road to conception. *F1000prime Rep.* (2013) 5:39. doi: 10.12703/P5-39
17. Sengupta P, Nwagha U, Dutta S, Krajewska-Kulak E, Izuka E. Evidence for decreasing sperm count in African population from 1965 to 2015. *Afr Health Sci.* (2017) 17:418–27. doi: 10.4314/ahs.v17i2.16
18. Levine H, Jorgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. *Hum Reprod Update.* (2017) 23:646–59. doi: 10.1093/humupd/dmx022
19. Jurewicz J, Radwan M, Sobala W, Ligocka D, Radwan P, Bochenek M, et al. Lifestyle and semen quality: role of modifiable risk factors. *Syst Biol Reprod Med.* (2014) 60:43–51. doi: 10.3109/19396368.2013.840687
20. Knez J. Endocrine-disrupting chemicals and male reproductive health. *Reprod Biomed Online.* (2013) 26:440–8. doi: 10.1016/j.rbmo.2013.02.005
21. Bonde JP. Occupational causes of male infertility. *Curr Opin Endocrinol Diab Obes.* (2013) 20:234–9. doi: 10.1097/MED.0b013e32835f3d4b
22. Sharma R, Biedenharn KR, Fedor JM, Agarwal A. Lifestyle factors and reproductive health: taking control of your fertility. *Reprod Biol Endocrinol.* (2013) 11:66. doi: 10.1186/1477-7827-11-66
23. Sharpe RM. Fetal/neonatal hormones and reproductive function of the male in adulthood. In: Wheeler T, Barker DJP, O'Brien PMS, Royal College of O, Gynaecologists, Programming RSGoF, editors. *Fetal Programming: Influences on Development and Disease in Later Life*. London: RCOG Press (1999). p. 187–94.
24. Papkoff H, Ekblad M. Ovine follicle stimulating hormone: preparation and characterization of its subunits. *Biochem Biophys Res Commun.* (1970) 40:614–21. doi: 10.1016/0006-291X(70)90948-4

25. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev.* (1997) 18:739–73. doi: 10.1210/er.18.6.739
26. Heckert LL, Griswold MD. The expression of the follicle-stimulating hormone receptor in spermatogenesis. *Recent Prog Horm Res.* (2002) 57:129–48. doi: 10.1210/rp.57.1.129
27. Heckert L, Griswold MD. Expression of the FSH receptor in the testis. *Recent Prog Horm Res.* (1993) 48:61–77. doi: 10.1016/B978-0-12-571148-7.50006-3
28. Griswold M, Mably E, Fritz IB. Stimulation by follicle stimulating hormone and dibutyryl cyclic AMP of incorporation of 3H-thymidine into nuclear DNA of cultured Sertoli cell-enriched preparations from immature rats. *Curr Top Mol Endocrinol.* (1975) 2:413–20. doi: 10.1007/978-1-4613-4440-7_29
29. Griswold MD, Mably ER, Fritz IB. FSH stimulation of DNA synthesis in Sertoli cells in culture. *Mol Cell Endocrinol.* (1976) 4:139–49. doi: 10.1016/0303-7207(76)90033-2
30. Orth JM, Higginbotham CA, Salisbury RL. Hemicastration causes and testosterone prevents enhanced uptake of [3H] thymidine by Sertoli cells in testes of immature rats. *Biol Reprod.* (1984) 30:263–70. doi: 10.1095/biolreprod30.1.263
31. Almiron I, Chemes H. Spermatogenic onset. II. FSH modulates mitotic activity of germ and Sertoli cells in immature rats. *Int J Androl.* (1988) 11:235–46. doi: 10.1111/j.1365-2605.1988.tb00998.x
32. Singh J, Handelsman DJ. The effects of recombinant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice. *J Androl.* (1996) 17:382–93.
33. Meachem SJ, McLachlan RI, de Kretser DM, Robertson DM, Wreford NG. Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. *Biol Reprod.* (1996) 54:36–44. doi: 10.1095/biolreprod54.1.36
34. Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, Allan CM. Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. *Endocrinology.* (2003) 144:509–17. doi: 10.1210/en.2002-220710
35. Allan CM, Garcia A, Spaliviero J, Zhang FP, Jimenez M, Huhtaniemi I, et al. Complete Sertoli cell proliferation induced by follicle-stimulating hormone (FSH) independently of luteinizing hormone activity: evidence from genetic models of isolated FSH action. *Endocrinology.* (2004) 145:1587–93. doi: 10.1210/en.2003-1164
36. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, et al. Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci USA.* (1998) 95:13612–7. doi: 10.1073/pnas.95.23.13612
37. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology.* (2000) 141:1795–803. doi: 10.1210/endo.141.5.7456
38. O'Shaughnessy PJ, Monteiro A, Abel M. Testicular development in mice lacking receptors for follicle stimulating hormone and androgen. *PLoS ONE.* (2012) 7:e35136. doi: 10.1371/journal.pone.0035136
39. Means AR, Huckins C. Coupled events in the early biochemical actions of FSH on the Sertoli cells of the testis. *Curr Top Mol Endocrinol.* (1974) 1:145–65. doi: 10.1007/978-1-4684-2595-6_8
40. Dattatreyamurty B, Figgs LW, Reichert LE Jr. Physical and functional association of follitropin receptors with cholera toxin-sensitive guanine nucleotide-binding protein. *J Biol Chem.* (1987) 262:11737–45.
41. Gloaguen P, Crepieux P, Heitzler D, Poupon A, Reiter E. Mapping the follicle-stimulating hormone-induced signaling networks. *Front Endocrinol.* (2011) 2:45. doi: 10.3389/fendo.2011.00045
42. Ulloa-Aguirre A, Reiter E, Crepieux P. FSH receptor signaling: complexity of interactions and signal diversity. *Endocrinology.* (2018) 159:3020–35. doi: 10.1210/en.2018-00452
43. Crepieux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, et al. The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. *Oncogene.* (2001) 20:4696–709. doi: 10.1038/sj.onc.1204632
44. Musnier A, Heitzler D, Boulo T, Tesseraud S, Durand G, Lecureuil C, et al. Developmental regulation of p70 S6 kinase by a G protein-coupled receptor dynamically modelized in primary cells. *Cell Mol Life Sci.* (2009) 66:3487–503. doi: 10.1007/s00018-009-0134-z
45. Riera MF, Regueira M, Galardo MN, Pellizzari EH, Meroni SB, Cigorraga SB. Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation. *Am J Physiol Endocrinol Metab.* (2012) 302:E914–23. doi: 10.1152/ajpendo.00477.2011
46. Walker WH, Fucci L, Habener JF. Expression of the gene encoding transcription factor cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein (CREB): regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells. *Endocrinology.* (1995) 136:3534–45. doi: 10.1210/endo.136.8.7628390
47. Delfino F, Walker WH. Stage-specific nuclear expression of NF-kappaB in mammalian testis. *Mol Endocrinol.* (1998) 12:1696–707.
48. Hamil KG, Conti M, Shimasaki S, Hall SH. Follicle-stimulating hormone regulation of AP-1: inhibition of c-jun and stimulation of jun-B gene transcription in the rat Sertoli cell. *Mol Cell Endocrinol.* (1994) 99:269–77. doi: 10.1016/0303-7207(94)90017-5
49. Lim K, Hwang BD. Follicle-stimulating hormone transiently induces expression of protooncogene c-myc in primary Sertoli cell cultures of early pubertal and prepubertal rat. *Mol Cell Endocrinol.* (1995) 111:51–6. doi: 10.1016/0303-7207(95)03543-G
50. Galardo MN, Gorga A, Merlo JP, Regueira M, Pellizzari EH, Cigorraga SB, et al. Participation of HIFs in the regulation of Sertoli cell lactate production. *Biochimie.* (2017) 132:9–18. doi: 10.1016/j.biochi.2016.10.006
51. Gorga A, Rindone G, Regueira M, Riera MF, Pellizzari EH, Cigorraga SB, et al. HIF involvement in the regulation of rat Sertoli cell proliferation by FSH. *Biochem Biophys Res Commun.* (2018) 502:508–14. doi: 10.1016/j.bbrc.2018.05.206
52. Kondo K, Kim WY, Lechpammer M, Kaelin WG Jr. Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol.* (2003) 1:E83. doi: 10.1371/journal.pbio.0000083
53. Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, et al. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol.* (2005) 25:5675–86. doi: 10.1128/MCB.25.13.5675-5686.2005
54. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol.* (2006) 7:85–96. doi: 10.1038/nrm1837
55. Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, et al. Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol.* (1996) 10:903–18.
56. Villalpando I, Lira E, Medina G, Garcia-Garcia E, Echeverria O. Insulin-like growth factor 1 is expressed in mouse developing testis and regulates somatic cell proliferation. *Exp Biol Med.* (2008) 233:419–26. doi: 10.3181/0708-RM-212
57. Borland K, Mita M, Oppenheimer CL, Blinderman LA, Massague J, Hall PF, et al. The actions of insulin-like growth factors I and II on cultured Sertoli cells. *Endocrinology.* (1984) 114:240–6. doi: 10.1210/endo-114-1-240
58. Oonk RB, Grootegoed JA. Insulin-like growth factor I (IGF-I) receptors on Sertoli cells from immature rats and age-dependent testicular binding of IGF-I and insulin. *Mol Cell Endocrinol.* (1988) 55:33–43. doi: 10.1016/0303-7207(88)90088-3
59. Jaillard C, Chatelain PG, Saez JM. *In vitro* regulation of pig Sertoli cell growth and function: effects of fibroblast growth factor and somatomedin-C. *Biol Reprod.* (1987) 37:665–74. doi: 10.1095/biolreprod37.3.665
60. Khan SA, Ndjountche L, Pratchard L, Spicer LJ, Davis JS. Follicle-stimulating hormone amplifies insulin-like growth factor I-mediated activation of AKT/protein kinase B signaling in immature rat Sertoli cells. *Endocrinology.* (2002) 143:2259–67. doi: 10.1210/endo.143.6.8838
61. Dance A, Thundathil J, Blondin P, Kastelic J. Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing postpubertal semen quality. *Theriogenology.* (2016) 86:687–94 e2. doi: 10.1016/j.theriogenology.2016.02.022
62. Froment P, Vigier M, Negre D, Fontaine I, Beghelli J, Cosset FL, et al. Inactivation of the IGF-I receptor gene in primary Sertoli cells

highlights the autocrine effects of IGF-I. *J Endocrinol.* (2007) 194:557–68. doi: 10.1677/JOE-07-0258
63. Saez JM, Chatelain PG, Perrard-Sapori MH, Jaillard C, Naville D. Differentiating effects of somatomedin-C/insulin-like growth factor I and insulin on Leydig and Sertoli cell functions. *Reprod Nutr Dev.* (1988) 28:989–1008. doi: 10.1051/rnd:19880701
64. Pitetti JL, Calvel P, Zimmermann C, Conne B, Papaioannou MD, Aubry F, et al. An essential role for insulin and IGF1 receptors in regulating sertoli cell proliferation, testis size, and FSH action in mice. *Mol Endocrinol.* (2013) 27:814–27. doi: 10.1210/me.2012-1258
65. Griffeth RJ, Carretero J, Burks DJ. Insulin receptor substrate 2 is required for testicular development. *PLoS ONE.* (2013) 8:e62103. doi: 10.1371/journal.pone.0062103
66. Naville D, Chatelain PG, Avallet O, Saez JM. Control of production of insulin-like growth factor I by pig Leydig and Sertoli cells cultured alone or together. Cell-cell interactions. *Mol Cell Endocrinol.* (1990) 70:217–24. doi: 10.1016/0303-7207(90)90212-Q
67. Yamamoto T, Nakayama Y, Abe SI. Mammalian follicle-stimulating hormone and insulin-like growth factor I (IGF-I) up-regulate IGF-I gene expression in organ culture of newt testis. *Mol Reprod Dev.* (2001) 60:56–64. doi: 10.1002/mrd.1061
68. Smith EP, Dickson BA, Chernausek SD. Insulin-like growth factor binding protein-3 secretion from cultured rat sertoli cells: dual regulation by follicle stimulating hormone and insulin-like growth factor-I. *Endocrinology.* (1990) 127:2744–51. doi: 10.1210/endo-127-6-2744
69. Rappaport MS, Smith EP. Insulin-like growth factor (IGF) binding protein 3 in the rat testis: follicle-stimulating hormone dependence of mRNA expression and inhibition of IGF-I action on cultured Sertoli cells. *Biol Reprod.* (1995) 52:419–25. doi: 10.1095/biolreprod52.2.419
70. Abel MH, Baker PJ, Charlton HM, Monteiro A, Verhoeven G, De Gendt K, et al. Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology.* (2008) 149:3279–85. doi: 10.1210/en.2008-0086
71. Sherwood OD. Relaxin's physiological roles and other diverse actions. *Endocr Rev.* (2004) 25:205–34. doi: 10.1210/er.2003-0013
72. Dschietzig T, Bartsch C, Baumann G, Stangl K. Relaxin-a pleiotropic hormone and its emerging role for experimental and clinical therapeutics. *Pharmacol Ther.* (2006) 112:38–56. doi: 10.1016/j.pharmthera.2006.03.004
73. Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, et al. Activation of orphan receptors by the hormone relaxin. *Science.* (2002) 295:671–4. doi: 10.1126/science.1065654
74. Cardoso LC, Nascimento AR, Royer C, Porto CS, Lazari MF. Locally produced relaxin may affect testis and vas deferens function in rats. *Reproduction.* (2010) 139:185–96. doi: 10.1530/REP-09-0146
75. Filonzi M, Cardoso LC, Pimenta MT, Queiroz DB, Avellar MC, Porto CS, et al. Relaxin family peptide receptors Rxfp1 and Rxfp2: mapping of the mRNA and protein distribution in the reproductive tract of the male rat. *Reprod Biol Endocrinol.* (2007) 5:29. doi: 10.1186/1477-7827-5-29
76. Nascimento AR, Pimenta MT, Lucas TF, Royer C, Porto CS, Lazari MF. Intracellular signaling pathways involved in the relaxin-induced proliferation of rat Sertoli cells. *Eur J Pharmacol.* (2012) 691:283–91. doi: 10.1016/j.ejphar.2012.07.021
77. Samuel CS, Zhao C, Yang Q, Wang H, Tian H, Tregear GW, et al. The relaxin gene knockout mouse: a model of progressive scleroderma. *J Invest Dermatol.* (2005) 125:692–9. doi: 10.1111/j.0022-202X.2005.23880.x
78. Nascimento AR, Pimenta MT, Lucas TF, Porto CS, Lazari MF. Relaxin and Sertoli cell proliferation. *Ital J Anat Embryol.* (2013) 118 (1 Suppl.):26–8. doi: 10.13128/IJAE-13885
79. Nascimento AR, Macheroni C, Lucas TF, Porto CS, Lazari MF. Crosstalk between FSH and relaxin at the end of the proliferative stage of rat Sertoli cells. *Reproduction.* (2016) 152:613–28. doi: 10.1530/REP-16-0330
80. Franchimont P, Demoulin A, Verstraelen-Proyard J, Hazee-Hagelstein MT, Tunbridge WM. Identification in human seminal fluid of an inhibin-like factor which selectively regulates FSH secretion. *J Reprod Fertil Suppl.* (1979) 26:123–33.
81. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, et al. A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem Biophys Res Commun.* (1986) 138:1129–37. doi: 10.1016/S0006-291X(86)80400-4
82. Barton DE, Yang-Feng TL, Mason AJ, Seeburg PH, Francke U. Mapping of genes for inhibin subunits alpha, beta A, and beta B on human and mouse chromosomes and studies of jsd mice. *Genomics.* (1989) 5:91–9. doi: 10.1016/0888-7543(89)90091-8
83. Hotten G, Neidhardt H, Schneider C, Pohl J. Cloning of a new member of the TGF-beta family: a putative new activin beta C chain. *Biochem Biophys Res Commun.* (1995) 206:608–13. doi: 10.1006/bbrc.1995.1086
84. Oda S, Nishimatsu S, Murakami K, Ueno N. Molecular cloning and functional analysis of a new activin beta subunit: a dorsal mesoderm-inducing activity in Xenopus. *Biochem Biophys Res Commun.* (1995) 210:581–8. doi: 10.1006/bbrc.1995.1699
85. Fang J, Yin W, Smiley E, Wang SQ, Bonadio J. Molecular cloning of the mouse activin beta E subunit gene. *Biochem Biophys Res Commun.* (1996) 228:669–74. doi: 10.1006/bbrc.1996.1715
86. Mason AJ, Hayflick JS, Ling N, Esch F, Ueno N, Ying SY, et al. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-beta. *Nature.* (1985) 318:659–63. doi: 10.1038/318659a0
87. Roberts V, Meunier H, Sawchenko PE, Vale W. Differential production and regulation of inhibin subunits in rat testicular cell types. *Endocrinology.* (1989) 125:2350–9. doi: 10.1210/endo-125-5-2350
88. de Winter JP, Vanderstichele HM, Verhoeven G, Timmerman MA, Wesseling JG, de Jong FH. Peritubular myoid cells from immature rat testes secrete activin-A and express activin receptor type II *in vitro*. *Endocrinology.* (1994) 135:759–67. doi: 10.1210/endo.135.2.8033824
89. Buzzard JJ, Farnworth PG, De Kretser DM, O'Connor AE, Wreford NG, Morrison JR. Proliferative phase sertoli cells display a developmentally regulated response to activin *in vitro*. *Endocrinology.* (2003) 144:474–83. doi: 10.1210/en.2002-220595
90. Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, Loveland KL. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. *Dev Biol.* (2000) 220:225–37. doi: 10.1006/dbio.2000.9625
91. Jeanes A, Wilhelm D, Wilson MJ, Bowles J, McClive PJ, Sinclair AH, et al. Evaluation of candidate markers for the peritubular myoid cell lineage in the developing mouse testis. *Reproduction.* (2005) 130:509–16. doi: 10.1530/rep.1.00718
92. Anderson RA, Cambray N, Hartley PS, McNeilly AS. Expression and localization of inhibin alpha, inhibin/activin betaA and betaB and the activin type II and inhibin beta-glycan receptors in the developing human testis. *Reproduction.* (2002) 123:779–88. doi: 10.1530/rep.0.1230779
93. Archambeault DR, Yao HH. Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. *Proc Natl Acad Sci USA.* (2010) 107:10526–31. doi: 10.1073/pnas.1000318107
94. Barakat B, O'Connor AE, Gold E, de Kretser DM, Loveland KL. Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction.* (2008) 136:345–59. doi: 10.1530/REP-08-0140
95. Fragale A, Puglisi R, Morena AR, Stefanini M, Boitani C. Age-dependent activin receptor expression pinpoints activin A as a physiological regulator of rat Sertoli cell proliferation. *Mol Hum Reprod.* (2001) 7:1107–14. doi: 10.1093/molehr/7.12.1107
96. Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. *Endocrinology.* (1995) 136:5438–44. doi: 10.1210/endo.136.12.7588293
97. Mendis SH, Meachem SJ, Sarraj MA, Loveland KL. Activin A balances Sertoli and germ cell proliferation in the fetal mouse testis. *Biol Reprod.* (2011) 84:379–91. doi: 10.1095/biolreprod.110.086231
98. Archambeault DR, Tomaszewski J, Childs AJ, Anderson RA, Yao HH. Testicular somatic cells, not gonocytes, are the major source of functional activin A during testis morphogenesis. *Endocrinology.* (2011) 152:4358–67. doi: 10.1210/en.2011-1288

99. Matzuk MM, Kumar TR, Bradley A. Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature.* (1995) 374:356–60. doi: 10.1038/374356a0
100. Miles DC, Wakeling SI, Stringer JM, van den Bergen JA, Wilhelm D, Sinclair AH, et al. Signaling through the TGF beta-activin receptors ALK4/5/7 regulates testis formation and male germ cell development. *PLoS ONE.* (2013) 8:e54606. doi: 10.1371/journal.pone.0054606
101. Itman C, Small C, Griswold M, Nagaraja AK, Matzuk MM, Brown CW, et al. Developmentally regulated SMAD2 and SMAD3 utilization directs activin signaling outcomes. *Dev Dynam.* (2009) 238:1688–700. doi: 10.1002/dvdy.21995
102. Nicholls PK, Stanton PG, Chen JL, Olcorn JS, Haverfield JT, Qian H, et al. Activin signaling regulates Sertoli cell differentiation and function. *Endocrinology.* (2012) 153:6065–77. doi: 10.1210/en.2012-1821
103. Schrewe H, Gendron-Maguire M, Harbison ML, Gridley T. Mice homozygous for a null mutation of activin beta B are viable and fertile. *Mechan Dev.* (1994) 47:43–51. doi: 10.1016/0925-4773(94)90094-9
104. Anawalt BD, Bebb RA, Matsumoto AM, Groome NP, Illingworth PJ, McNeilly AS, et al. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *J Clin Endocrinol Metab.* (1996) 81:3341–5.
105. Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature.* (1992) 360:313–9. doi: 10.1038/360313a0
106. Matzuk MM, Finegold MJ, Mather JP, Krummen L, Lu H, Bradley A. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. *Proc Natl Acad Sci USA.* (1994) 91:8817–21. doi: 10.1073/pnas.91.19.8817
107. Li Q, Graff JM, O'Connor AE, Loveland KL, Matzuk MM. SMAD3 regulates gonadal tumorigenesis. *Mol Endocrinol.* (2007) 21:2472–86. doi: 10.1210/me.2007-0147
108. Looyenga BD, Hammer GD. Genetic removal of Smad3 from inhibin-null mice attenuates tumor progression by uncoupling extracellular mitogenic signals from the cell cycle machinery. *Mol Endocrinol.* (2007) 21:2440–57. doi: 10.1210/me.2006-0402
109. Gnessi L, Fabbri A, Spera G. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. *Endocr Rev.* (1997) 18:541–609. doi: 10.1210/er.18.4.541
110. Loveland KL, Klein B, Pueschl D, Indumathy S, Bergmann M, Loveland BE, et al. Cytokines in male fertility and reproductive pathologies: immunoregulation and beyond. *Front Endocrinol.* (2017) 8:307. doi: 10.3389/fendo.2017.00307
111. Gerard N, Syed V, Bardin W, Genetet N, Jegou B. Sertoli cells are the site of interleukin-1 alpha synthesis in rat testis. *Mol Cell Endocrinol.* (1991) 82:R13–6. doi: 10.1016/0303-7207(91)90019-O
112. Wang DL, Nagpal ML, Calkins JH, Chang WW, Sigel MM, Lin T. Interleukin-1 beta induces interleukin-1 alpha messenger ribonucleic acid expression in primary cultures of Leydig cells. *Endocrinology.* (1991) 129:2862–6. doi: 10.1210/endo-129-6-2862
113. Lin T, Wang D, Nagpal ML. Human chorionic gonadotropin induces interleukin-1 gene expression in rat Leydig cells *in vivo*. *Mol Cell Endocrinol.* (1993) 95:139–45. doi: 10.1016/0303-7207(93)90039-M
114. Hayes R, Chalmers SA, Nikolic-Paterson DJ, Atkins RC, Hedger MP. Secretion of bioactive interleukin 1 by rat testicular macrophages *in vitro*. *J Androl.* (1996) 17:41–9.
115. Hayrabedyan S, Todorova K, Jabeen A, Metodieva G, Toshkov S, Metodiev MV, et al. Sertoli cells have a functional NALP3 inflammasome that can modulate autophagy and cytokine production. *Sci Rep.* (2016) 6:18896. doi: 10.1038/srep18896
116. Cudicini C, Lejeune H, Gomez E, Bosmans E, Ballet F, Saez J, et al. Human Leydig cells and Sertoli cells are producers of interleukins-1 and−6. *J Clin Endocrinol Metab.* (1997) 82:1426–33. doi: 10.1210/jc.82.5.1426
117. Gomez E, Morel G, Cavalier A, Lienard MO, Haour F, Courtens JL, et al. Type I and type II interleukin-1 receptor expression in rat, mouse, and human testes. *Biol Reprod.* (1997) 56:1513–26. doi: 10.1095/biolreprod56.6.1513
118. Petersen C, Boitani C, Froysa B, Soder O. Interleukin-1 is a potent growth factor for immature rat sertoli cells. *Mol Cell Endocrinol.* (2002) 186:37–47. doi: 10.1016/S0303-7207(01)00680-3
119. Petersen C, Svechnikov K, Froysa B, Soder O. The p38 MAPK pathway mediates interleukin-1-induced Sertoli cell proliferation. *Cytokine.* (2005) 32:51–9. doi: 10.1016/j.cyto.2005.07.014
120. Jonsson CK, Zetterstrom RH, Holst M, Parvinen M, Soder O. Constitutive expression of interleukin-1alpha messenger ribonucleic acid in rat Sertoli cells is dependent upon interaction with germ cells. *Endocrinology.* (1999) 140:3755–61. doi: 10.1210/endo.140.8.6900
121. De SK, Chen HL, Pace JL, Hunt JS, Terranova PF, Enders GC. Expression of tumor necrosis factor-alpha in mouse spermatogenic cells. *Endocrinology.* (1993) 133:389–96. doi: 10.1210/endo.133.1.8319585
122. Mauduit C, Besset V, Caussanel V, Benahmed M. Tumor necrosis factor alpha receptor p55 is under hormonal (follicle-stimulating hormone) control in testicular Sertoli cells. *Biochem Biophys Res Commun.* (1996) 224:631–7. doi: 10.1006/bbrc.1996.1077
123. Petersen C, Froysa B, Soder O. Endotoxin and proinflammatory cytokines modulate Sertoli cell proliferation *in vitro*. *J Reprod Immunol.* (2004) 61:13–30. doi: 10.1016/j.jri.2003.10.003
124. Sharpe RM. *Regulation of Spermatogenesis*. Knobil E, Neill JD, editors. New York, NY: Raven Press (1994).
125. McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, et al. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog Horm Res.* (2002) 57:149–79. doi: 10.1210/rp.57.1.149
126. Griswold SL, Behringer RR. Fetal Leydig cell origin and development. *Sex Dev.* (2009) 3:1–15. doi: 10.1159/000200077
127. Corbier P, Edwards DA, Roffi J. The neonatal testosterone surge: a comparative study. *Arch Int Physiol Biochim Biophys.* (1992) 100:127–31. doi: 10.3109/13813459209035274
128. Ketelslegers JM, Hetzel WD, Sherins RJ, Catt KJ. Developmental changes in testicular gonadotropin receptors: plasma gonadotropins and plasma testosterone in the rat. *Endocrinology.* (1978) 103:212–22. doi: 10.1210/endo-103-1-212
129. Forest MG, Cathiard AM. Pattern of plasma testosterone and delta4-androstenedione in normal newborns: evidence for testicular activity at birth. *J Clin Endocrinol Metab.* (1975) 41:977–80. doi: 10.1210/jcem-41-5-977
130. Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. *Endocr Rev.* (2007) 28:778–808. doi: 10.1210/er.2007-0019
131. Bremner WJ, Millar MR, Sharpe RM, Saunders PT. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology.* (1994) 135:1227–34. doi: 10.1210/endo.135.3.8070367
132. Grootegoed JA, Peters MJ, Mulder E, Rommerts FF, Van der Molen HJ. Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. *Mol Cell Endocrinol.* (1977) 9:159–67. doi: 10.1016/0303-7207(77)90117-4
133. Anthony CT, Kovacs WJ, Skinner MK. Analysis of the androgen receptor in isolated testicular cell types with a microassay that uses an affinity ligand. *Endocrinology.* (1989) 125:2628–35. doi: 10.1210/endo-125-5-2628
134. Pelletier G, Labrie C, Labrie F. Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. *J Endocrinol.* (2000) 165:359–70. doi: 10.1677/joe.0.1650359
135. Vornberger W, Prins G, Musto NA, Suarez-Quian CA. Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology.* (1994) 134:2307–16. doi: 10.1210/endo.134.5.8156934
136. Zhou X, Kudo A, Kawakami H, Hirano H. Immunohistochemical localization of androgen receptor in mouse testicular germ cells during fetal and postnatal development. *Anat Rec.* (1996) 245:509–18. doi: 10.1002/(SICI)1097-0185(199607)245:3<509::AID-AR7>3.0.CO;2-M
137. Merlet J, Racine C, Moreau E, Moreno SG, Habert R. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *Proc Natl Acad Sci USA.* (2007) 104:3615–20. doi: 10.1073/pnas.0611421104
138. Buzek SW, Sanborn BM. Increase in testicular androgen receptor during sexual maturation in the rat. *Biol Reprod.* (1988) 39:39–49. doi: 10.1095/biolreprod39.1.39

139. You L, Sar M. Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. *Endocrine.* (1998) 9:253–61. doi: 10.1385/ENDO:9:3:253
140. Zhou Q, Shima JE, Nie R, Friel PJ, Griswold MD. Androgen-regulated transcripts in the neonatal mouse testis as determined through microarray analysis. *Biol Reprod.* (2005) 72:1010–9. doi: 10.1095/biolreprod.104.035915
141. Johnston H, Baker PJ, Abel M, Charlton HM, Jackson G, Fleming L, et al. Regulation of Sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse. *Endocrinology.* (2004) 145:318–29. doi: 10.1210/en.2003-1055
142. Atanassova NN, Walker M, McKinnell C, Fisher JS, Sharpe RM. Evidence that androgens and oestrogens, as well as follicle-stimulating hormone, can alter Sertoli cell number in the neonatal rat. *J Endocrinol.* (2005) 184:107–17. doi: 10.1677/joe.1.05884
143. Tan KA, De Gendt K, Atanassova N, Walker M, Sharpe RM, Saunders PT, et al. The role of androgens in sertoli cell proliferation and functional maturation: studies in mice with total or Sertoli cell-selective ablation of the androgen receptor. *Endocrinology.* (2005) 146:2674–83. doi: 10.1210/en.2004-1630
144. Skinner MK, Fritz IB. Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. *Proc Natl Acad Sci USA.* (1985) 82:114–8. doi: 10.1073/pnas.82.1.114
145. Swinnen K, Cailleau J, Heyns W, Verhoeven G. Prostatic stromal cells and testicular peritubular cells produce similar paracrine mediators of androgen action. *Endocrinology.* (1990) 126:142–50. doi: 10.1210/endo-126-1-142
146. Zhang C, Yeh S, Chen YT, Wu CC, Chuang KH, Lin HY, et al. Oligozoospermia with normal fertility in male mice lacking the androgen receptor in testis peritubular myoid cells. *Proc Natl Acad Sci USA.* (2006) 103:17718–23. doi: 10.1073/pnas.0608556103
147. Hazra R, Corcoran L, Robson M, McTavish KJ, Upton D, Handelsman DJ, et al. Temporal role of Sertoli cell androgen receptor expression in spermatogenic development. *Mol Endocrinol.* (2013) 27:12–24. doi: 10.1210/me.2012-1219
148. Buzzard JJ, Wreford NG, Morrison JR. Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat sertoli cells. *Endocrinology.* (2003) 144:3722–31. doi: 10.1210/en.2003-0379
149. Fix C, Jordan C, Cano P, Walker WH. Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. *Proc Natl Acad Sci USA.* (2004) 101:10919–24. doi: 10.1073/pnas.0404278101
150. Cheng J, Watkins SC, Walker WH. Testosterone activates mitogen-activated protein kinase via Src kinase and the epidermal growth factor receptor in sertoli cells. *Endocrinology.* (2007) 148:2066–74. doi: 10.1210/en.2006-1465
151. Berg AH, Rice CD, Rahman MS, Dong J, Thomas P. Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: I. Discovery in female atlantic croaker and evidence ZIP9 mediates testosterone-induced apoptosis of ovarian follicle cells. *Endocrinology.* (2014) 155:4237–49. doi: 10.1210/en.2014-1198
152. Bulldan A, Dietze R, Shihan M, Scheiner-Bobis G. Non-classical testosterone signaling mediated through ZIP9 stimulates claudin expression and tight junction formation in Sertoli cells. *Cell Signal.* (2016) 28:1075–85. doi: 10.1016/j.cellsig.2016.04.015
153. Harvey CB, Williams GR. Mechanism of thyroid hormone action. *Thyroid.* (2002) 12:441–6. doi: 10.1089/105072502760143791
154. Yen PM. Physiological and molecular basis of thyroid hormone action. *Physiol Rev.* (2001) 81:1097–142. doi: 10.1152/physrev.2001.81.3.1097
155. Wrutniak C, Cassar-Malek I, Marchal S, Rascle A, Heusser S, Keller JM, et al. A 43-kDa protein related to c-Erb A alpha 1 is located in the mitochondrial matrix of rat liver. *J Biol Chem.* (1995) 270:16347–54. doi: 10.1074/jbc.270.27.16347
156. Casas F, Rochard P, Rodier A, Cassar-Malek I, Marchal-Victorion S, Wiesner RJ, et al. A variant form of the nuclear triiodothyronine receptor c-ErbAalpha1 plays a direct role in regulation of mitochondrial RNA synthesis. *Mol Cell Biol.* (1999) 19:7913–24. doi: 10.1128/MCB.19.12.7913
157. Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S, et al. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology.* (2005) 146:2864–71. doi: 10.1210/en.2005-0102
158. Buzzard JJ, Morrison JR, O'Bryan MK, Song Q, Wreford NG. Developmental expression of thyroid hormone receptors in the rat testis. *Biol Reprod.* (2000) 62:664–9. doi: 10.1095/biolreprod62.3.664
159. Palmero S, Trucchi P, Prati M, Fugassa E, Lanni A, Goglia F. Effect of thyroid status on the oxidative capacity of Sertoli cells isolated from immature rat testis. *Eur J Endocrinol.* (1994) 130:308–12. doi: 10.1530/eje.0.1300308
160. Zanatta AP, Zanatta L, Goncalves R, Zamoner A, Silva FR. Rapid responses to reverse T(3) hormone in immature rat Sertoli cells: calcium uptake and exocytosis mediated by integrin. *PLoS ONE.* (2013) 8:e77176. doi: 10.1371/journal.pone.0077176
161. Cooke PS, Meisami E. Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. *Endocrinology.* (1991) 129:237–43. doi: 10.1210/endo-129-1-237
162. De Franca LR, Hess RA, Cooke PS, Russell LD. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. *Anat Rec.* (1995) 242:57–69. doi: 10.1002/ar.1092420108
163. van Haaster LH, de Jong FH, Docter R, de Rooij DG. High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. *Endocrinology.* (1993) 133:755–60. doi: 10.1210/endo.133.2.8344214
164. Auharek SA, de Franca LR. Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL/6J mice made transiently hypo- and hyperthyroidic during the neonatal period. *J Anat.* (2010) 216:577–88. doi: 10.1111/j.1469-7580.2010.01219.x
165. Cooke PS, Zhao YD, Bunick D. Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. *Biol Reprod.* (1994) 51:1000–5. doi: 10.1095/biolreprod51.5.1000
166. Palmero S, Prati M, Bolla F, Fugassa E. Tri-iodothyronine directly affects rat Sertoli cell proliferation and differentiation. *J Endocrinol.* (1995) 145:355–62. doi: 10.1677/joe.0.1450355
167. Holsberger DR, Buchold GM, Leal MC, Kiesewetter SE, O'Brien DA, Hess RA, et al. Cell-cycle inhibitors p27Kip1 and p21Cip1 regulate murine Sertoli cell proliferation. *Biol Reprod.* (2005) 72:1429–36. doi: 10.1095/biolreprod.105.040386
168. Arambepola NK, Bunick D, Cooke PS. Thyroid hormone and follicle-stimulating hormone regulate Mullerian-inhibiting substance messenger ribonucleic acid expression in cultured neonatal rat Sertoli cells. *Endocrinology.* (1998) 139:4489–95. doi: 10.1210/endo.139.11.6315
169. Arambepola NK, Bunick D, Cooke PS. Thyroid hormone effects on androgen receptor messenger RNA expression in rat Sertoli and peritubular cells. *J Endocrinol.* (1998) 156:43–50. doi: 10.1677/joe.0.1560043
170. Palmero S, De Marco P, Fugassa E. Thyroid hormone receptor beta mRNA expression in Sertoli cells isolated from prepubertal testis. *J Mol Endocrinol.* (1995) 14:131–4. doi: 10.1677/jme.0.0140131
171. Ulisse S, Jannini EA, Carosa E, Piersanti D, Graziano FM, D'Armiento M. Inhibition of aromatase activity in rat Sertoli cells by thyroid hormone. *J Endocrinol.* (1994) 140:431–6. doi: 10.1677/joe.0.1400431
172. Holsberger DR, Kiesewetter SE, Cooke PS. Regulation of neonatal Sertoli cell development by thyroid hormone receptor alpha1. *Biol Reprod.* (2005) 73:396–403. doi: 10.1095/biolreprod.105.041426
173. Fumel B, Guerquin MJ, Livera G, Staub C, Magistrini M, Gauthier C, et al. Thyroid hormone limits postnatal Sertoli cell proliferation *in vivo* by activation of its alpha1 isoform receptor (TRalpha1) present in these cells and by regulation of Cdk4/JunD/c-myc mRNA levels in mice. *Biol Reprod.* (2012) 87:16, 1–9. doi: 10.1095/biolreprod.111.098418
174. Fumel B, Roy S, Fouchecourt S, Livera G, Parent AS, Casas F, et al. Depletion of the p43 mitochondrial T3 receptor increases Sertoli cell proliferation in mice. *PLoS ONE.* (2013) 8:e74015. doi: 10.1371/journal.pone.0074015
175. Gilleron J, Nebout M, Scarabelli L, Senegas-Balas F, Palmero S, Segretain D, et al. A potential novel mechanism involving connexin 43 gap junction for control of sertoli cell proliferation by thyroid hormones. *J Cell Physiol.* (2006) 209:153–61. doi: 10.1002/jcp.20716

176. Sridharan S, Simon L, Meling DD, Cyr DG, Gutstein DE, Fishman GI, et al. Proliferation of adult sertoli cells following conditional knockout of the Gap junctional protein GJA1 (connexin 43) in mice. *Biol Reprod.* (2007) 76:804–12. doi: 10.1095/biolreprod.106.059212
177. Beumer TL, Kiyokawa H, Roepers-Gajadien HL, van den Bos LA, Lock TM, Gademan IS, et al. Regulatory role of p27kip1 in the mouse and human testis. *Endocrinology.* (1999) 140:1834–40. doi: 10.1210/endo.140.4.6638
178. Holsberger DR, Jirawatnotai S, Kiyokawa H, Cooke PS. Thyroid hormone regulates the cell cycle inhibitor p27Kip1 in postnatal murine Sertoli cells. *Endocrinology.* (2003) 144:3732–8. doi: 10.1210/en.2003-0389
179. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* (1998) 67:425–79. doi: 10.1146/annurev.biochem.67.1.425
180. Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, Hershko A. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J Biol Chem.* (2003) 278:25752–7. doi: 10.1074/jbc.M301774200
181. Sun Y, Yang W, Luo H, Wang X, Chen Z, Zhang J, et al. Thyroid hormone inhibits the proliferation of piglet Sertoli cell via PI3K signaling pathway. *Theriogenology.* (2015) 83:86–94. doi: 10.1016/j.theriogenology.2014.08.003
182. Imamura K, Ogura T, Kishimoto A, Kaminishi M, Esumi H. Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator, 5-aminoimidazole- 4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem Biophys Res Commun.* (2001) 287:562–7. doi: 10.1006/bbrc.2001.5627
183. Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation *in vitro* and *in vivo* via AMP-activated protein kinase. *J Biol Chem.* (2005) 280:39582–93. doi: 10.1074/jbc.M507443200
184. Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol.* (2009) 196:65–80. doi: 10.1111/j.1748-1716.2009.01972.x
185. Yamauchi M, Kambe F, Cao X, Lu X, Kozaki Y, Oiso Y, et al. Thyroid hormone activates adenosine 5'-monophosphate-activated protein kinase via intracellular calcium mobilization and activation of calcium/calmodulin-dependent protein kinase kinase-beta. *Mol Endocrinol.* (2008) 22:893–903. doi: 10.1210/me.2007-0249
186. Irrcher I, Walkinshaw DR, Sheehan TE, Hood DA. Thyroid hormone (T3) rapidly activates p38 and AMPK in skeletal muscle *in vivo*. *J Appl Physiol.* (2008) 104:178–85. doi: 10.1152/japplphysiol.00643.2007
187. Videla LA, Fernandez V, Cornejo P, Vargas R, Morales P, Ceballo J, et al. T(3)-induced liver AMP-activated protein kinase signaling: redox dependency and upregulation of downstream targets. *World J Gastroenterol.* (2014) 20:17416–25. doi: 10.3748/wjg.v20.i46.17416
188. McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR, et al. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. *Mol Cell Biol.* (2003) 23:38–54. doi: 10.1128/MCB.23.1.38-54.2003
189. Gorga A, Rindone GM, Regueira M, Pellizzari EH, Camberos MC, Cigorraga SB, et al. Effect of resveratrol on Sertoli cell proliferation. *J Cell Biochem.* (2018) 119:10131–42. doi: 10.1002/jcb.27350
190. Singh BK, Sinha RA, Zhou J, Xie SY, You SH, Gauthier K, et al. FoxO1 deacetylation regulates thyroid hormone-induced transcription of key hepatic gluconeogenic genes. *J Biol Chem.* (2013) 288:30365–72. doi: 10.1074/jbc.M113.504845
191. Thakran S, Sharma P, Attia RR, Hori RT, Deng X, Elam MB, et al. Role of sirtuin 1 in the regulation of hepatic gene expression by thyroid hormone. *J Biol Chem.* (2013) 288:807–18. doi: 10.1074/jbc.M112.437970
192. Vazquez-Anaya G, Martinez B, Sonanez-Organis JG, Nakano D, Nishiyama A, Ortiz RM. Exogenous thyroxine improves glucose intolerance in insulin-resistant rats. *J Endocrinol.* (2017) 232:501–11. doi: 10.1530/JOE-16-0428
193. Hess RA, Carnes K. The role of estrogen in testis and the male reproductive tract: a review and species comparison. *Anim Reprod.* (2004) 1:5–30.
194. Carreau S, Hess RA. Oestrogens and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci.* (2010) 365:1517–35. doi: 10.1098/rstb.2009.0235
195. Santen RJ, Brodie H, Simpson ER, Siiteri PK, Brodie A. History of aromatase: saga of an important biological mediator and therapeutic target. *Endocr Rev.* (2009) 30:343–75. doi: 10.1210/er.2008-0016
196. Free MJ, Jaffe RA. Collection of rete testis fluid from rats without previous efferent duct ligation. *Biol Reprod.* (1979) 20:269–78. doi: 10.1095/biolreprod20.2.269
197. Tsai-Morris CH, Aquilano DR, Dufau ML. Cellular localization of rat testicular aromatase activity during development. *Endocrinology.* (1985) 116:38–46. doi: 10.1210/endo-116-1-38
198. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, et al. Germ cells of the mouse testis express P450 aromatase. *Endocrinology.* (1993) 132:1396–401. doi: 10.1210/endo.132.3.8440194
199. Papadopoulos V, Carreau S, Szerman-Joly E, Drosdowsky MA, Dehennin L, Scholler R. Rat testis 17 beta-estradiol: identification by gas chromatography-mass spectrometry and age related cellular distribution. *J Steroid Biochem.* (1986) 24:1211–6. doi: 10.1016/0022-4731(86)90385-7
200. Rappaport MS, Smith EP. Insulin-like growth factor I inhibits aromatization induced by follice-stimulating hormone in rat sertoli cell culture. *Biol Reprod.* (1996) 54:446–52. doi: 10.1095/biolreprod54.2.446
201. Le Magueresse B, Jegou B. *In vitro* effects of germ cells on the secretory activity of Sertoli cells recovered from rats of different ages. *Endocrinology.* (1988) 122:1672–80. doi: 10.1210/endo-122-4-1672
202. Levin ER. Plasma membrane estrogen receptors. *Trends Endocrinol Metab.* (2009) 20:477–82. doi: 10.1016/j.tem.2009.06.009
203. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc Natl Acad Sci USA.* (2006) 103:9063–8. doi: 10.1073/pnas.0603339103
204. Li L, Hisamoto K, Kim KH, Haynes MP, Bauer PM, Sanjay A, et al. Variant estrogen receptor-c-Src molecular interdependence and c-Src structural requirements for endothelial NO synthase activation. *Proc Natl Acad Sci USA.* (2007) 104:16468–73. doi: 10.1073/pnas.0704315104
205. Chimento A, Sirianni R, Casaburi I, Pezzi V. GPER Signaling in Spermatogenesis and Testicular Tumors. *Front Endocrinol.* (2014) 5:30. doi: 10.3389/fendo.2014.00030
206. Evinger AJ III, Levin ER. Requirements for estrogen receptor alpha membrane localization and function. *Steroids.* (2005) 70:361–3. doi: 10.1016/j.steroids.2005.02.015
207. Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, Sharpe RM. Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J Endocrinol.* (1997) 153:485–95. doi: 10.1677/joe.0.1530485
208. van Pelt AM, de Rooij DG, van der Burg B, van der Saag PT, Gustafsson JA, Kuiper GG. Ontogeny of estrogen receptor-beta expression in rat testis. *Endocrinology.* (1999) 140:478–83. doi: 10.1210/endo.140.1.6438
209. Bois C, Delalande C, Nurmio M, Parvinen M, Zanatta L, Toppari J, et al. Age- and cell-related gene expression of aromatase and estrogen receptors in the rat testis. *J Mol Endocrinol.* (2010) 45:147–59. doi: 10.1677/JME-10-0041
210. Lucas TFG, Lazari MFM, Porto CS. Differential role of the estrogen receptors ESR1 and ESR2 on the regulation of proteins involved with proliferation and differentiation of Sertoli cells from 15-day-old rats. *Mol Cell Endocrinol.* (2014) 382:84–96. doi: 10.1016/j.mce.2013.09.015
211. Olde B, Leeb-Lundberg LM. GPR30/GPER1: searching for a role in estrogen physiology. *Trends Endocrinol Metab.* (2009) 20:409–16. doi: 10.1016/j.tem.2009.04.006
212. Otto C, Fuchs I, Kauselmann G, Kern H, Zevnik B, Andreasen P, et al. GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biol Reprod.* (2009) 80:34–41. doi: 10.1095/biolreprod.108.071175
213. Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D, et al. Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology.* (2009) 150:1722–30. doi: 10.1210/en.2008-1488
214. Sirianni R, Chimento A, Ruggiero C, De Luca A, Lappano R, Ando S, et al. The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17beta-estradiol on mouse spermatogonial GC-1 cell line. *Endocrinology.* (2008) 149:5043–51. doi: 10.1210/en.2007-1593
215. Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Ando S, et al. 17 beta-estradiol activates rapid signaling pathways involved in rat pachytene

spermatocytes apoptosis through GPR30 and ER alpha. *Mol Cell Endocrinol.* (2010) 320:136–44. doi: 10.1016/j.mce.2010.01.035
216. Lucas TF, Royer C, Siu ER, Lazari MF, Porto CS. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat sertoli cells. *Biol Reprod.* (2010) 83:307–17. doi: 10.1095/biolreprod.110.084160
217. Lucas TF, Pimenta MT, Pisolato R, Lazari MF, Porto CS. 17beta-estradiol signaling and regulation of Sertoli cell function. *Spermatogenesis.* (2011) 1:318–24. doi: 10.4161/spmg.1.4.18903
218. Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, et al. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. *Endocrinology.* (1999) 140:5364–73. doi: 10.1210/endo.140.11.7108
219. Berger T, Kentfield L, Roser JF, Conley A. Stimulation of Sertoli cell proliferation: defining the response interval to an inhibitor of estrogen synthesis in the boar. *Reproduction.* (2012) 143:523–9. doi: 10.1530/REP-11-0464
220. Berger T, Conley AJ, Van Klompenberg M, Roser JF, Hovey RC. Increased testicular Sertoli cell population induced by an estrogen receptor antagonist. *Mol Cell Endocrinol.* (2013) 366:53–8. doi: 10.1016/j.mce.2012.11.011
221. Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, et al. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinology.* (2001) 142:2435–42. doi: 10.1210/endo.142.6.8211
222. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, et al. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci USA.* (1999) 96:7986–91. doi: 10.1073/pnas.96.14.7986
223. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA.* (1993) 90:11162–6. doi: 10.1073/pnas.90.23.11162
224. Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, et al. Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res.* (1996) 51:159–86; discussion 86–8.
225. Gould ML, Hurst PR, Nicholson HD. The effects of oestrogen receptors alpha and beta on testicular cell number and steroidogenesis in mice. *Reproduction.* (2007) 134:271–9. doi: 10.1530/REP-07-0025
226. Antal MC, Krust A, Chambon P, Mark M. Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant. *Proc Natl Acad Sci USA.* (2008) 105:2433–8. doi: 10.1073/pnas.0712029105
227. Yang WR, Zhu FW, Zhang JJ, Wang Y, Zhang JH, Lu C, et al. PI3K/Akt activated by GPR30 and Src regulates 17beta-estradiol-induced cultured immature boar sertoli cells proliferation. *Reprod Sci.* (2017) 24:57–66. doi: 10.1177/1933719116649696
228. Cavazzini D, Galdieri M, Ottonello S. Retinoic acid synthesis in the somatic cells of rat seminiferous tubules. *Biochim Biophys Acta.* (1996) 1313:139–45. doi: 10.1016/0167-4889(96)00065-1
229. Deltour L, Haselbeck RJ, Ang HL, Duester G. Localization of class I and class IV alcohol dehydrogenases in mouse testis and epididymis: potential retinol dehydrogenases for endogenous retinoic acid synthesis. *Biol Reprod.* (1997) 56:102–9. doi: 10.1095/biolreprod56.1.102
230. Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, et al. Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology.* (2006) 147:96–110. doi: 10.1210/en.2005-0953
231. Guo X, Morris P, Gudas L. Follicle-stimulating hormone and leukemia inhibitory factor regulate Sertoli cell retinol metabolism. *Endocrinology.* (2001) 142:1024–32. doi: 10.1210/endo.142.3.7996
232. Kurlandsky SB, Gamble MV, Ramakrishnan R, Blaner WS. Plasma delivery of retinoic acid to tissues in the rat. *J Biol Chem.* (1995) 270:17850–7. doi: 10.1074/jbc.270.30.17850
233. Raverdeau M, Gely-Pernot A, Feret B, Dennefeld C, Benoit G, Davidson I, et al. Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *Proc Natl Acad Sci USA.* (2012) 109:16582–7. doi: 10.1073/pnas.1214936109
234. Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W. Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. *Acta Anatom.* (1979) 103:159–68. doi: 10.1159/000145007
235. Sobhon P, Mitranond V, Tosukhowong P, Chindaduangrat W. Cytological changes in the testes of vitamin-A-deficient rats. II. Ultrastructural study of the seminiferous tubules. *Acta Anatom.* (1979) 103:169–83. doi: 10.1159/000145008
236. van Pelt AM, de Rooij DG. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biol Reprod.* (1990) 43:363–67. doi: 10.1095/biolreprod43.3.363
237. Hogarth CA, Griswold MD. The key role of vitamin A in spermatogenesis. *J Clin Invest.* (2010) 120:956–62. doi: 10.1172/JCI41303
238. Akmal KM, Dufour JM, Kim KH. Retinoic acid receptor alpha gene expression in the rat testis: potential role during the prophase of meiosis and in the transition from round to elongating spermatids. *Biol Reprod.* (1997) 56:549–56. doi: 10.1095/biolreprod56.2.549
239. Dufour JM, Kim KH. Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. *Biol Reprod.* (1999) 61:1300–8. doi: 10.1095/biolreprod61.5.1300
240. Boulogne B, Levacher C, Durand P, Habert R. Retinoic acid receptors and retinoid X receptors in the rat testis during fetal and postnatal development: immunolocalization and implication in the control of the number of gonocytes. *Biol Reprod.* (1999) 61:1548–57. doi: 10.1095/biolreprod61.6.1548
241. Nicholls PK, Harrison CA, Rainczuk KE, Wayne Vogl A, Stanton PG. Retinoic acid promotes Sertoli cell differentiation and antagonises activin-induced proliferation. *Mol Cell Endocrinol.* (2013) 377:33–43. doi: 10.1016/j.mce.2013.06.034
242. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, et al. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci USA.* (1993) 90:7225–9. doi: 10.1073/pnas.90.15.7225
243. Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. *Cell.* (1993) 73:643–58. doi: 10.1016/0092-8674(93)90246-M
244. Gely-Pernot A, Raverdeau M, Celebi C, Dennefeld C, Feret B, Klopfenstein M, et al. Spermatogonia differentiation requires retinoic acid receptor gamma. *Endocrinology.* (2012) 153:438–49. doi: 10.1210/en.2011-1102
245. Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, et al. Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev.* (1996) 10:80–92. doi: 10.1101/gad.10.1.80
246. Luo J, Pasceri P, Conlon RA, Rossant J, Giguere V. Mice lacking all isoforms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. *Mech Dev.* (1995) 53:61–71. doi: 10.1016/0925-4773(95)00424-6
247. Krezel W, Dupe V, Mark M, Dierich A, Kastner P, Chambon P. RXR gamma null mice are apparently normal and compound RXR alpha +/-/RXR beta -/-/RXR gamma -/- mutant mice are viable. *Proc Natl Acad Sci USA.* (1996) 93:9010–4. doi: 10.1073/pnas.93.17.9010
248. Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D, et al. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell.* (1994) 78:987–1003. doi: 10.1016/0092-8674(94)90274-7
249. Vernet N, Dennefeld C, Guillou F, Chambon P, Ghyselinck NB, Mark M. Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *EMBO J.* (2006) 25:5816–25. doi: 10.1038/sj.emboj.7601447
250. Hasegawa K, Saga Y. Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression. *Development.* (2012) 139:4347–55. doi: 10.1242/dev.080119
251. Huang HF, Yang CS, Meyenhofer M, Gould S, Boccabella AV. Disruption of sustentacular (Sertoli) cell tight junctions and regression of spermatogenesis in vitamin-A-deficient rats. *Acta Anatom.* (1988) 133:10–5. doi: 10.1159/000146606
252. Morales A, Cavicchia JC. Spermatogenesis and blood-testis barrier in rats after long-term Vitamin A deprivation. *Tissue Cell.* (2002) 34:349–55. doi: 10.1016/S0040816602000356

253. Chung SS, Choi C, Wang X, Hallock L, Wolgemuth DJ. Aberrant distribution of junctional complex components in retinoic acid receptor alpha-deficient mice. *Microscopy Res Tech.* (2010) 73:583–96. doi: 10.1002/jemt.20797
254. Tsong SD, Phillips D, Halmi N, Liotta AS, Margioris A, Bardin CW, et al. ACTH and beta-endorphin-related peptides are present in multiple sites in the reproductive tract of the male rat. *Endocrinology.* (1982) 110:2204–6. doi: 10.1210/endo-110-6-2204
255. Bardin CW, Shaha C, Mather J, Salomon Y, Margioris AN, Liotta AS, et al. Identification and possible function of pro-opiomelanocortin-derived peptides in the testis. *Ann N Y Acad Sci.* (1984) 438:346–64. doi: 10.1111/j.1749-6632.1984.tb38296.x
256. Fabbri A, Knox G, Buczko E, Dufau ML. Beta-endorphin production by the fetal Leydig cell: regulation and implications for paracrine control of Sertoli cell function. *Endocrinology.* (1988) 122:749–55. doi: 10.1210/endo-122-2-749
257. Fabbri A, Tsai-Morris CH, Luna S, Fraioli F, Dufau ML. Opiate receptors are present in the rat testis. Identification and localization in Sertoli cells. *Endocrinology.* (1985) 117:2544–6. doi: 10.1210/endo-117-6-2544
258. Jenab S, Morris PL. Interleukin-6 regulation of kappa opioid receptor gene expression in primary sertoli cells. *Endocrine.* (2000) 13:11–5. doi: 10.1385/ENDO:13:1:11
259. Orth JM. FSH-induced Sertoli cell proliferation in the developing rat is modified by beta-endorphin produced in the testis. *Endocrinology.* (1986) 119:1876–8. doi: 10.1210/endo-119-4-1876
260. Orth JM, Boehm R. Endorphin suppresses FSH-stimulated proliferation of isolated neonatal Sertoli cells by a pertussis toxin-sensitive mechanism. *Anat Rec.* (1990) 226:320–7. doi: 10.1002/ar.1092260308
261. da Silva VA, Jr., Vieira AC, Pinto CF, de Paula TA, Palma MB, Lins Amorim MJ, et al. Neonatal treatment with naloxone increases the population of Sertoli cells and sperm production in adult rats. *Reprod Nutr Dev.* (2006) 46:157–66. doi: 10.1051/rnd:2006001
262. Fairley KF, Barrie JU, Johnson W. Sterility and testicular atrophy related to cyclophosphamide therapy. *Lancet.* (1972) 1:568–9. doi: 10.1016/S0140-6736(72)90358-3
263. Qureshi MS, Pennington JH, Goldsmith HJ, Cox PE. Cyclophosphamide therapy and sterility. *Lancet.* (1972) 2:1290–1. doi: 10.1016/S0140-6736(72)92657-8
264. Meistrich ML. Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertil Steril.* (2013) 100:1180–6. doi: 10.1016/j.fertnstert.2013.08.010
265. Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. *JAMA.* (1988) 259:2123–5. doi: 10.1001/jama.1988.03720140043031
266. Nurmio M, Keros V, Lahteenmaki P, Salmi T, Kallajoki M, Jahnukainen K. Effect of childhood acute lymphoblastic leukemia therapy on spermatogonia populations and future fertility. *J Clin Endocrinol Metab.* (2009) 94:2119–22. doi: 10.1210/jc.2009-0060
267. Wasilewski-Masker K, Seidel KD, Leisenring W, Mertens AC, Shnorhavorian M, Ritenour CW, et al. Male infertility in long-term survivors of pediatric cancer: a report from the childhood cancer survivor study. *J Cancer Survivorship.* (2014) 8:437–47. doi: 10.1007/s11764-014-0354-6
268. Chow EJ, Stratton KL, Leisenring WM, Oeffinger KC, Sklar CA, Donaldson SS, et al. Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor Study cohort. *Lancet Oncol.* (2016) 17:567–76. doi: 10.1016/S1470-2045(16)00086-3
269. Hou M, Chrysis D, Nurmio M, Parvinen M, Eksborg S, Soder O, et al. Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity. *Cancer Res.* (2005) 65:9999–10005. doi: 10.1158/0008-5472.CAN-05-2004
270. Tremblay AR, Delbes G. *In vitro* study of doxorubicin-induced oxidative stress in spermatogonia and immature Sertoli cells. *Toxicol Appl Pharmacol.* (2018) 348:32–42. doi: 10.1016/j.taap.2018.04.014
271. Nurmio M, Toppari J, Kallio J, Hou M, Soder O, Jahnukainen K. Functional *in vitro* model to examine cancer therapy cytotoxicity in maturing rat testis. *Reprod Toxicol.* (2009) 27:28–34. doi: 10.1016/j.reprotox.2008.10.004
272. Liu F, Li XL, Lin T, He DW, Wei GH, Liu JH, et al. The cyclophosphamide metabolite, acrolein, induces cytoskeletal changes and oxidative stress in Sertoli cells. *Mol Biol Rep.* (2012) 39:493–500. doi: 10.1007/s11033-011-0763-9
273. Smart E, Lopes F, Rice S, Nagy B, Anderson RA, Mitchell RT, et al. Chemotherapy drugs cyclophosphamide, cisplatin and doxorubicin induce germ cell loss in an in vitro model of the prepubertal testis. *Sci Rep.* (2018) 8:1773. doi: 10.1038/s41598-018-19761-9
274. Faqi AS, Klug A, Merker HJ, Chahoud I. Ganciclovir induces reproductive hazards in male rats after short-term exposure. *Hum Exp Toxicol.* (1997) 16:505–11. doi: 10.1177/096032719701600905
275. Narayana K. A purine nucleoside analogue-acyclovir [9-(2-hydroxyethoxymethyl)-9h-guanine] reversibly impairs testicular functions in mouse. *J Toxicol Sci.* (2008) 33:61–70. doi: 10.2131/jts.33.61
276. Nihi F, Moreira D, Santos Lourenco AC, Gomes C, Araujo SL, Zaia RM, et al. Testicular effects following *in utero* exposure to the antivirals acyclovir and ganciclovir in rats. *Toxicol Sci.* (2014) 139:220–33. doi: 10.1093/toxsci/kfu024
277. Qiu R, Horvath A, Stahlmann R. Effects of four nucleoside analogues used as antiviral agents on rat Sertoli cells (SerW3) *in vitro*. *Arch Toxicol.* (2016) 90:1975–81. doi: 10.1007/s00204-016-1743-6
278. Ben Maamar M, Lesne L, Hennig K, Desdoits-Lethimonier C, Kilcoyne KR, Coiffec I, et al. Ibuprofen results in alterations of human fetal testis development. *Sci Rep.* (2017) 7:44184. doi: 10.1038/srep44184
279. Rossitto M, Marchive C, Pruvost A, Sellem E, Ghettas A, Badiou S, et al. Intergenerational effects on mouse sperm quality after in utero exposure to acetaminophen and ibuprofen. *FASEB J.* (2019) 33:339–57. doi: 10.1096/fj.201800488RRR
280. Romero R, Erez O, Huttemann M, Maymon E, Panaitescu B, Conde-Agudelo A, et al. Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity. *Am J Obstetr Gynecol.* (2017) 217:282–302. doi: 10.1016/j.ajog.2017.06.003
281. Moghetti P, Castello R, Negri C, Tosi F, Perrone F, Caputo M, et al. Metformin effects on clinical features, endocrine and metabolic profiles, and insulin sensitivity in polycystic ovary syndrome: a randomized, double-blind, placebo-controlled 6-month trial, followed by open, long-term clinical evaluation. *J Clin Endocrinol Metab.* (2000) 85:139–46. doi: 10.1210/jcem.85.1.6293
282. Vanky E, Zahlsen K, Spigset O, Carlsen SM. Placental passage of metformin in women with polycystic ovary syndrome. *Fertil Steril.* (2005) 83:1575–8. doi: 10.1016/j.fertnstert.2004.11.051
283. Brufani C, Crino A, Fintini D, Patera PI, Cappa M, Manco M. Systematic review of metformin use in obese nondiabetic children and adolescents. *Horm Res Paediatr.* (2013) 80:78–85. doi: 10.1159/000353760
284. Adeyemo MA, McDuffie JR, Kozlosky M, Krakoff J, Calis KA, Brady SM, et al. Effects of metformin on energy intake and satiety in obese children. *Diabetes Obes Metab.* (2015) 17:363–70. doi: 10.1111/dom.12426
285. Smith JD, Mills E, Carlisle SE. Treatment of Pediatric Type 2 Diabetes. *Ann Pharmacother.* (2016) 50:768–77. doi: 10.1177/1060028016655179
286. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, et al. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cell Mol Life Sci.* (2007) 64:1290–302. doi: 10.1007/s00018-007-7080-4
287. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin D1 level. *Oncogene.* (2008) 27:3576–86. doi: 10.1038/sj.onc.1211024
288. Rattan R, Giri S, Hartmann LC, Shridhar V. Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. *J Cell Mol Med.* (2011) 15:166–78. doi: 10.1111/j.1582-4934.2009.00954.x
289. Tartarin P, Moison D, Guibert E, Dupont J, Habert R, Rouiller-Fabre V, et al. Metformin exposure affects human and mouse fetal testicular cells. *Hum Reprod.* (2012) 27:3304–14. doi: 10.1093/humrep/des264
290. Faure M, Guibert E, Alves S, Pain B, Rame C, Dupont J, et al. The insulin sensitiser metformin regulates chicken Sertoli and germ cell populations. *Reproduction.* (2016) 151:527–38. doi: 10.1530/REP-15-0565
291. Rindone GM, Gorga A, Regueira M, Pellizzari EH, Cigorraga SB, Galardo MN, et al. Metformin counteracts the effects of FSH on rat Sertoli cell proliferation. *Reproduction.* (2018) 156:93–101. doi: 10.1530/REP-18-0233

292. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature.* (2014) 510:542–6. doi: 10.1038/nature13270
293. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 348 (Pt 3):607–14. doi: 10.1042/bj3480607
294. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clin Sci.* (2012) 122:253–70. doi: 10.1042/CS20110386
295. Garcia MS, Constantino DH, Silva AP, Perobelli JE. Fish pollutants MeHg and Aroclor cause permanent structural damage in male gonads and kidneys after prepubertal exposure. *Int J Exp Pathol.* (2016) 97:360–8. doi: 10.1111/iep.12200
296. Mylchreest E, Cattley RC, Foster PM. Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci.* (1998) 43:47–60. doi: 10.1093/toxsci/43.1.47
297. Wisniewski P, Romano RM, Kizys MM, Oliveira KC, Kasamatsu T, Giannocco G, et al. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. *Toxicology.* (2015) 329:1–9. doi: 10.1016/j.tox.2015.01.002
298. Wilker C, Johnson L, Safe S. Effects of developmental exposure to indole-3-carbinol or 2,3,7,8-tetrachlorodibenzo-p-dioxin on reproductive potential of male rat offspring. *Toxicol. Appl Pharmacol.* (1996) 141:68–75. doi: 10.1016/S0041-008X(96)80010-X
299. Axelstad M, Hass U, Scholze M, Christiansen S, Kortenkamp A, Boberg J. EDC IMPACT: reduced sperm counts in rats exposed to human relevant mixtures of endocrine disrupters. *Endocr Connect.* (2018) 7:139–48. doi: 10.1530/EC-17-0307
300. Sekaran S, Balaganapathy P, Parsanathan R, Elangovan S, Gunashekar J, Bhat FA, et al. Lactational exposure of phthalate causes long-term disruption in testicular architecture by altering tight junctional and apoptotic protein expression in Sertoli cells of first filial generation pubertal Wistar rats. *Hum Exp Toxicol.* (2015) 34:575–90. doi: 10.1177/0960327114555926
301. Sedha S, Kumar S, Shukla S. Role of oxidative stress in male reproductive dysfunctions with reference to phthalate compounds. *Urol J.* (2015) 12:2304–16. doi: 10.22037/uj.v12i5.3009
302. Cheng CY, Wong EW, Lie PP, Li MW, Su L, Siu ER, et al. Environmental toxicants and male reproductive function. *Spermatogenesis.* (2011) 1:2–13. doi: 10.4161/spmg.1.1.13971
303. Yao PL, Lin YC, Sawhney P, Richburg JH. Transcriptional regulation of FasL expression and participation of sTNF-alpha in response to sertoli cell injury. *J Biol Chem.* (2007) 282:5420–31. doi: 10.1074/jbc.M609068200
304. Wang M, Su P. The role of the Fas/FasL signaling pathway in environmental toxicant-induced testicular cell apoptosis: an update. *Syst Biol Reprod Med.* (2018) 64:93–102. doi: 10.1080/19396368.2017.1422046
305. Li LH, Jester WF Jr, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol.* (1998) 153:258–65. doi: 10.1006/taap.1998.8550
306. Li LH, Jester WF Jr, Laslett AL, Orth JM. A single dose of Di-(2-ethylhexyl) phthalate in neonatal rats alters gonocytes, reduces sertoli cell proliferation, and decreases cyclin D2 expression. *Toxicol Appl Pharmacol.* (2000) 166:222–9. doi: 10.1006/taap.2000.8972
307. Zhang L, Gao M, Zhang T, Chong T, Wang Z, Zhai X, et al. Protective effects of genistein against Mono-(2-ethylhexyl) phthalate-induced oxidative damage in prepubertal sertoli cells. *Biomed Res Int.* (2017) 2017:2032697. doi: 10.1155/2017/2032697
308. Yin X, Ma T, Han R, Ding J, Zhang H, Han X, et al. MiR-301b-3p/3584–5p enhances low-dose mono-n-butyl phthalate (MBP)-induced proliferation by targeting Rasd1 in Sertoli cells. *Toxicol In Vitro.* (2018) 47:79–88. doi: 10.1016/j.tiv.2017.11.009
309. Salian S, Doshi T, Vanage G. Perinatal exposure of rats to bisphenol A affects fertility of male offspring–an overview. *Reprod Toxicol.* (2011) 31:359–62. doi: 10.1016/j.reprotox.2010.10.008
310. Ge LC, Chen ZJ, Liu H, Zhang KS, Su Q, Ma XY, et al. Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: a comparative proteomic analysis. *Biochim Biophys Acta.* (2014) 1840:2663–73. doi: 10.1016/j.bbagen.2014.05.018
311. Johnson L, Staub C, Silge RL, Harris MW, Chapin RE. The pesticide methoxychlor given orally during the perinatal/juvenile period, reduced the spermatogenic potential of males as adults by reducing their Sertoli cell number. *Reprod Nutr Dev.* (2002) 42:573–80. doi: 10.1051/rnd:2002043
312. Wang BJ, Zheng WL, Feng NN, Wang T, Zou H, Gu JH, et al. The effects of autophagy and PI3K/AKT/m-TOR signaling pathway on the cell-cycle arrest of rats primary sertoli cells induced by zearalenone. *Toxins.* (2018) 10:398. doi: 10.3390/toxins10100398
313. Ness DK, Schantz SL, Moshtaghian J, Hansen LG. Effects of perinatal exposure to specific PCB congeners on thyroid hormone concentrations and thyroid histology in the rat. *Toxicol Lett.* (1993) 68:311–23. doi: 10.1016/0378-4274(93)90023-Q
314. Cooke PS, Zhao YD, Hansen LG. Neonatal polychlorinated biphenyl treatment increases adult testis size and sperm production in the rat. *Toxicol Appl Pharmacol.* (1996) 136:112–7. doi: 10.1006/taap.1996.0013
315. Kim IS. Effects of exposure of lactating female rats to polychlorinated biphenyls (Pcbs) on testis weight, sperm production and sertoli cell numbers in the adult male offspring. *J Vet Med Sci.* (2001) 63:5–9. doi: 10.1292/jvms.63.5
316. Fiorini C, Tilloy-Ellul A, Chevalier S, Charuel C, Pointis G. Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. *Reprod Toxicol.* (2004) 18:413–21. doi: 10.1016/j.reprotox.2004.01.002
317. Aravindakshan J, Cyr DG. Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway. *Biol Reprod.* (2005) 72:1232–40. doi: 10.1095/biolreprod.104.038596
318. Pointis G, Gilleron J, Carette D, Segretain D. Testicular connexin 43, a precocious molecular target for the effect of environmental toxicants on male fertility. *Spermatogenesis.* (2011) 1:303–17. doi: 10.4161/spmg.1.4.18392

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 17 April 2019
doi: 10.3389/fendo.2019.00248

Distinct Expression Patterns of Osteopontin and Dentin Matrix Protein 1 Genes in Pituitary Gonadotrophs

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Cell-matrix interactions play important roles in pituitary development, physiology, and pathogenesis. In other tissues, a family of non-collagenous proteins, termed SIBLINGs, are known to contribute to cell-matrix interactions. Anterior pituitary gland expresses two SIBLING genes, *Dmp1* (dentin matrix protein-1) and *Spp1* (secreted phosphoprotein-1) encoding DMP1 and osteopontin proteins, respectively, but their expression pattern and roles in pituitary functions have not been clarified. Here we provide novel evidence supporting the conclusion that *Spp1*/osteopontin, like *Dmp1*/DMP1, are expressed in gonadotrophs in a sex- and age-specific manner. Other anterior pituitary cell types do not express these genes. In contrast to *Dmp1*, *Spp1* expression is higher in males; in females, the expression reaches the peak during the diestrus phase of estrous cycle. In further contrast to *Dmp1* and marker genes for gonadotrophs, the expression of *Spp1* is not regulated by gonadotropin-releasing hormone *in vivo* and *in vitro*. However, *Spp1* expression increases progressively after pituitary cell dispersion in both female and male cultures. We may speculate that gonadotrophs signal to other pituitary cell types about changes in the structure of pituitary cell-matrix network by osteopontin, a function consistent with the role of this secretory protein in postnatal tissue remodeling, extracellular matrix reorganization after injury, and tumorigenesis.

Keywords: pituitary, gonadotrophs, SIBLINGs, *Spp1*, osteopontin, *Dmp1*, GnRH, cell-matrix

OPEN ACCESS

Edited by:
László Hunyady,
Semmelweis University, Hungary

Reviewed by:
John Chang,
University of Alberta, Canada
Yves Combarnous,
Centre National de la Recherche Scientifique (CNRS), France

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 February 2019*
Accepted: *29 March 2019*
Published: *17 April 2019*

Citation:
Bjelobaba I, Janjic MM, Prévide RM, Abebe D, Kucka M and Stojilkovic SS (2019) Distinct Expression Patterns of Osteopontin and Dentin Matrix Protein 1 Genes in Pituitary Gonadotrophs. Front. Endocrinol. 10:248. doi: 10.3389/fendo.2019.00248

INTRODUCTION

Cell—extracellular matrix (ECM) tridimensional network is critical for the proper functioning of all tissues (1), including anterior pituitary gland (2). Individual components of ECM include two main classes of macromolecules; proteoglycans and fibrous proteins (laminin, collagens, elastins, and fibronectin) (3, 4). The effects of the ECM are mediated mainly by plasma membrane receptors called integrins; individual components of ECM bind to different integrins, leading to activation of multiple signaling pathways (5). In anterior pituitary, the presence of ECM molecules, like collagens, laminin, and small leucine-rich proteoglycans, and cell types producing these proteins have been identified (6–10). Pituitary cells also express integrins (11). There is increasing evidence that ECM is critical for development and differentiation of the pituitary gland (12), for postnatal cell migration and pituitary remodeling (13), and for hormone secretion (14, 15). As in other tissues, ECM molecules may also have important roles in pituitary tumorigenesis (16).

In addition to proteoglycans and fibrous proteins, ECM contains other proteins, including SIBLINGs (Small Integrin-Binding Ligand, N-linked Glycophosphoproteins). SIBLINGs are encoded by a family of five genes, comprising secreted phosphoprotein 1 (*Spp1*), which encodes osteopontin (OPN), integrin-binding sialoprotein, which encodes bone sialoprotein, and dentin matrix protein 1 (*Dmp1*), dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein, which encode proteins with the same name (17). SIBLINGs are soluble, secreted proteins that can act as modulators of cell adhesion as well as autocrine and paracrine ligands for ECM receptors. For example, OPN activates a variety of integrin receptors as well as CD44 receptor splice variants (18). The ligand activities of SIBLINGs are modulated by post-translational modifications, such as phosphorylation, glycosylation, proteolytic processing, sulphation, and transglutaminase cross-linking (19, 20).

SIBLINGs were initially described as mineralized tissue-associated genes (21). However, recent findings indicate that they are more widely distributed, including normal ductal epithelia in salivary gland (22) and kidney (23). *Spp1*/OPN were also detected in central nervous system (24), where they may play a role in neurodegenerative diseases, such as Alzheimer's disease (25), Parkinson's disease (26), and multiple sclerosis (27, 28). *Dmp1*/DMP1 was reported to be expressed in the brain, as well as in the liver, muscle, pancreas and kidney (29). SIBLING gene family is also expressed in various tumors (18, 30) and OPN was suggested to be a valuable biomarker for diagnosing and treating cancers (31).

Our recent RNA-sequence analysis revealed that *Dmp1* and *Spp1*, but no other SIBLING genes, were also expressed in anterior pituitary cells (32). The expression of *Dmp1* is restricted to gonadotrophs, cells that produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and is stimulated by gonadotropin-releasing hormone (GnRH) but not by other hypothalamic releasing factors. GnRH-induced expression of this gene is coupled with release of DMP1 in extracellular medium through the regulated secretory pathway. *In vivo*, the sex-specific pituitary *Dmp1* expression is established during the peripubertal period and is elevated after ovulation. GnRH induction of *Dmp1* is mediated by the protein kinase C signaling pathway through ERK1/2 signaling pathway; in addition, the response is facilitated by progesterone (32). It has also been shown that *Spp1* is expressed in gonadotrophs and that mRNA levels were down regulated in anterior pituitary of lactating animals and by injection of estradiol (33).

Here we summarize work on *Spp1* expression in rat anterior pituitary cells *in vivo* and *in vitro*. These include sexual dimorphism in *Spp1* expression during maturation, effects of cell-matrix network destruction by cell dispersion procedure on *Spp1* expression, and evaluation of the role of GnRH receptors (GnRHR) in the expression of this gene. We also studied the expression pattern of OPN in prepubertal females and males and cycling females as well as the cell type specificity in expression of this protein. Finally, we compared *Spp1* expression with *Dmp1* expression in pituitary gonadotrophs.

Abbreviations: DMP1, dentin matrix protein 1; ECM, extracellular matrix; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; LH, luteinizing hormone; OPN, osteopontin; SIBLINGs, small integrin-binding ligand, N-linked glycophosphoproteins; *Spp1*, secreted phosphoprotein 1.

METHODS

Animals

Experiments were performed with female and male Sprague Dawley rats obtained from Taconic Farms (Germantown, NY). Animals were housed under constant conditions of temperature and humidity, with light on between 6 a.m. and 8 p.m. All experiments were repeated at least three times and were approved by the NICHD Animal Care and Use Committee (16-041).

Ontogeny of *Spp1*/OPN Expression

Experiments were performed with 2 days to 12 weeks old female and male rats. In some postpubertal females, a vaginal smear was taken of adult females to obtain information about the estrous cycle stage. Vaginal material was stained by a 0.1% aqueous solution of methylene blue and examined under a microscope. Animals were euthanized via asphyxiation with CO_2 and whole pituitary or anterior pituitary glands were removed and used for histological preparations or RNA extraction as described below.

Anterior Pituitary Cell Culture

For *in vitro* experiments, 4- or 7-week-old female and male rats were euthanized in the morning. After decapitation anterior pituitary glands were removed and pituitary cells were mechanically dispersed after trypsin and EDTA treatments as previously described (34). Dispersed cells were seeded on poly-D-lysine coated 24-well plates, 1.5 million per well. Plated cells were initially cultured in medium 199 containing Earle's salts, sodium bicarbonate, penicillin (100 units per ml), streptomycin (100 μg per ml) and 10% heat-inactivated horse serum (Life Technologies, Grand Island, NY). If not otherwise specified, experiments were performed with cells cultured overnight, washed and bathed in medium 199 with Hank's salt and containing 0.1% BSA. At the end of experiments, attached cells were scraped for RNA extraction.

In Vivo Treatments

Four- or seven-week-old female and male rats were injected once intraperitoneally with a GnRHR agonist, buserelin acetate (5 μg/0.4 ml/per animal) from Sigma (St. Louis, MO) or PBS (0.4 ml/per animal). Euthanasia was performed 3, 6, or 9 h after intraperitoneal injections. After decapitation, blood was collected, and serum was separated and stored at −80°C for LH concentration measurement. The whole anterior pituitaries were collected in RNA later stabilization solution (Thermo Fisher Scientific, Waltham, MA) for RNA extraction.

qRT-PCR Analysis

Total RNA was extracted from individual anterior pituitary glands and primary cultures of anterior pituitary cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with a Transcriptor First Stand cDNA

Synthesis Kit (Roche Applied Sciences, Indianapolis, IN). Quantitative RT-PCR was performed using Applied Biosystems pre-designed Taq-Man Gene Expression Assays for rats using the LightCycler® TaqMan® Master Mix and the LightCycler 2.0 Real-time PCR system (Roche Applied Science). Target gene expression levels were determined by the comparative 2^(-delta C(T)) quantification method using *Gapdh* as the reference gene, which was previously established to be a suitable reference gene for the anterior pituitary tissue (35). Applied Biosystems predesigned TaqMan Gene Expression Assays were used: *Dmp1*: Rn01450122_m1, *Spp1* (Rn00681031_m1), *Gnrhr* (Rn00578981_m1), and *Gapdh*: Rn01462662_g1.

Immunohistochemical Analysis

Whole pituitaries were quickly and carefully isolated and fixed in Bouin's solution for 48 h. Tissue was then embedded in paraffin and cut in coronal plane. Five μm thick sections were mounted on glass slides and processed for immunohistochemistry as previously described (36, 37). Briefly, after deparaffinization, antigen retrieval in citrate buffer (0.01 M, pH 6) was performed. Monoclonal OPN antibody (The Developmental Studies Hybridoma Bank, Iowa City, IA) in 1:400 dilution was applied overnight at 4°C. Secondary donkey anti-mouse-HRP (Santa Cruz Biotechnology, Dallas, TX) was then applied at 1:200 dilution for 2 h, and visualization was afterwards performed with diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA). Slides were mounted with DPX (Sigma, St. Louis, MO) and sections examined under an Olympus BX61 microscope. For double immunofluorescence studies, after incubation of sections with the OPN antibody, secondary Alexa Fluor donkey-anti-mouse 488 (Thermo Fisher Scientific, Waltham, MA) was applied at 1:400 dilution for 2 h. Sections were then incubated for 2 h with rabbit-anti rat LH or guinea pig-anti FSH (1:500 dilution) obtained from Dr. A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Following the incubation with donkey-anti rabbit or donkey-anti guinea pig 555 Alexa Fluor secondary antibodies (1:400 dilution) slides were mounted with Mowiol based mounting medium and examined under inverted Zeiss LSM 510 confocal microscope. Triple immunofluorescence labeling was done as described above using Alexa Fluor Dyes: donkey-anti rabbit 488, donkey-anti guinea pig 555, and donkey-anti mouse 647 (Thermo Fisher Scientific, Waltham, MA), and the sections were examined under Leica TCS SP5 II confocal microscope.

Statistics

All numerical values in the text are reported as the mean ± SEM from one of at least three similar *in vivo* or *in vitro* experiments. KaleidaGraph Program (Synergy Software, Reading, Pennsylvania) was used for all calculation and graph presentation. Significant differences between means were determined by a Student's *t*-test or an ANOVA accompanied with the *post hoc* Student-Newman-Keuls test as well as for regression/correlation analyses and calculation of the half time of decay in gene expression. *P*-values of <0.05 were considered significant.

RESULTS

Spp1 Is Expressed in Anterior Pituitary of Developing Animals in a Sex-Specific Manner

The *Spp1* expression was investigated in male and female anterior pituitary tissue from 2 days to 12 weeks old rats (**Figure 1A**). During this period, the gene was expressed in both sexes and the mRNA expression varied between 5 and 70% of the expression of *Gapdh*, a housekeeping gene. Pituitaries obtained from animals up to 3 weeks of age showed no sex difference in the expression of *Spp1*. From week four onward, however, the sex-specific expression pattern was established. First, *Spp1* levels were always significantly higher in male pituitaries. Second, there were differences in terms of timing needed to reach the peak in mRNA expression. In males, there was a progressive increase in *Spp1* expression, reaching the peak value at 5 weeks of age, with ∼13-fold increase in expression when compared to the second day of age. This was followed by a progressive decrease in gene expression during peripubertal and postpubertal periods. In females, however, the first peak in expression was reached during infantile period, with an ∼3-fold increase compared to the second day of age. This was followed by a gradual decline during the juvenile, peripubertal and postpubertal age, with a secondary transient increase in expression at the age of 8 weeks (**Figure 1A**).

In both female and male rats, the developmental profiles of *Spp1* were highly comparable to profiles of major gonadotroph-specific (hereafter marker) genes, *Gnrhr, Lhb*, and *Fshb*, as well as to the gonadotroph/thyrotroph-specific gene *Cga* (35). This prompted us to examine the relationship between *Spp1* expression vs. marker gene expression during sexual maturation. This was done using a linear correlation analysis and the Pearson *r*-coefficient as an indicator of significance of correlation. Scatter data points for *Gnrhr* vs. *Spp1* in developing males (**Figure 1B**) and females (**Figure 1C**), had a linear tendency, with the *r* value significant in both cases.

Furthermore, *Spp1* expression correlated well with the expression of *Lhb*, *Fshb*, and *Cga* in males (**Figures 2A–C**). There was also correlation between *Spp1* vs. *Cga* in females (**Figure 2F**), with comparable *r*-coefficient values in females and males, as well as between *Spp1* and *Lhb* expression (**Figure 2D**), but with lower *r*-coefficient in females when compared with males. Finally, the *r*-coefficient value was not significant when *Spp1* expression was compared with *Fshb* expression in females (**Figure 2E**). The data points from prepubertal females (shown in gray) and in postpubertal females (shown in white) suggest that correlation was attenuated in postpubertal animals.

To clarify how the estrous cycle influences gene expression, we examined *Lhb*, *Fshb*, *Cga*, and *Spp1* expression in pituitaries from proestrus, estrus, and combined diestrus-1 (metestrus) and diestrus-2 animals. The expression of *Lhb*, *Fshb*, and *Cga* was highest in diestrus animals and lowest in estrus animals (**Figure 2G**). The pattern of *Spp1* expression during of estrous cycle was different: the smallest was during proestrus (2.84 ± 0.70, $n = 14$), followed by estrus (7.50 ± 0.89, $n = 8$; $P < 0.01$

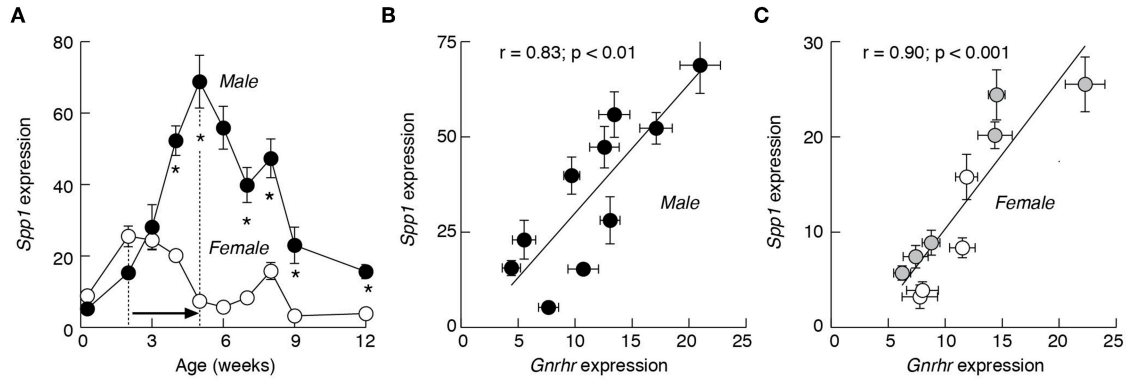

FIGURE 1 | The expression pattern of *Spp1* mRNA in anterior pituitary of developing rats is comparable to *Gnrhr* expression. **(A)** The sex-specific developmental profiles of *Spp1* expression. White circles: females; black circles: males. In postpubertal females, the mean values are derived from regularly cycling animals in proestrus, estrus, and diestrus 1 and 2 stages of the cycle. **(B,C)** The expression of *Spp1* correlates with expression of *Gnrhr*, a gonadotroph marker gene, in male **(B)** and female **(C)** pituitaries. Data points shown are mean ± SEM values from 6 to 37 animals per group, relative to *Gapdh* (set as 100%). Correlation and linear regression analyses and statistical evaluation are described in Material and Methods; *r*, coefficient of correlation. The mean ± SEM values for *Gnrhr* are derived from (38). Asterisks indicate significant differences between pairs **(A)**; the *p* values for *r* coefficient are shown on top of panels **(B,C)**. Gray circles, prepubertal females; white circle, postpubertal females **(C)**.

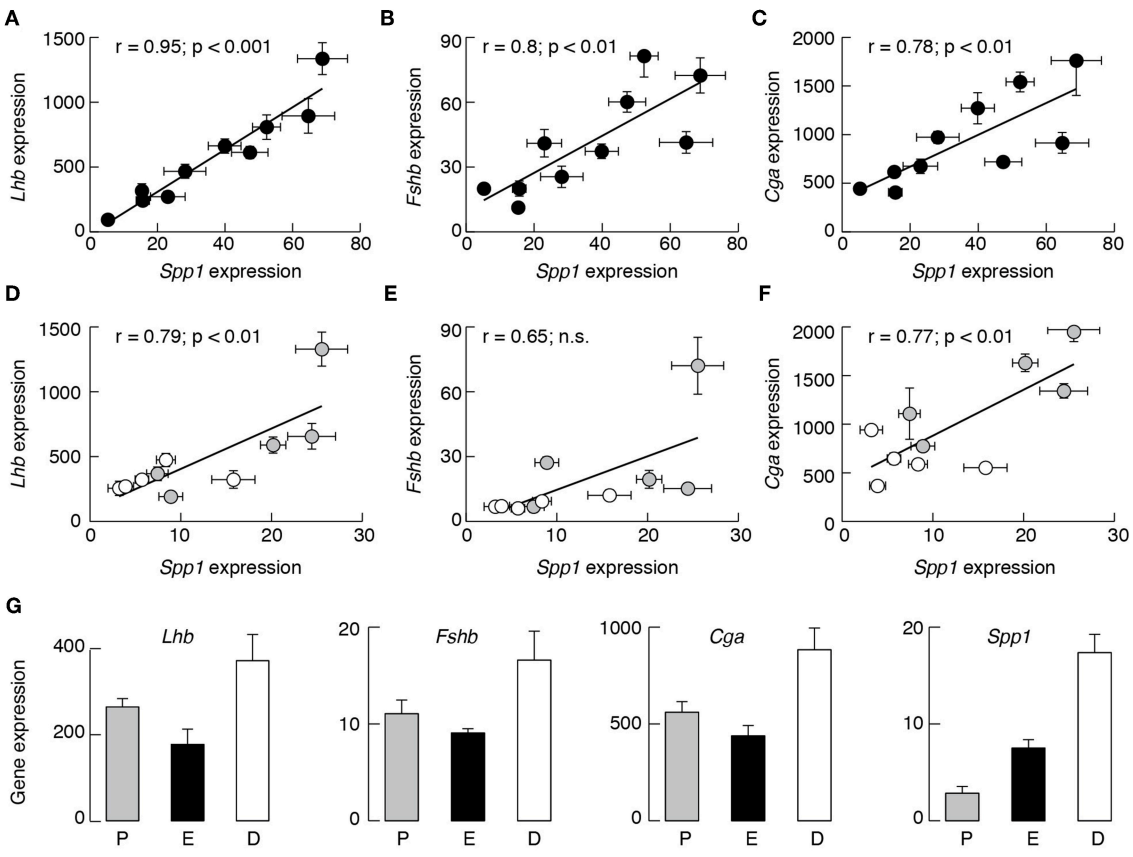

FIGURE 2 | The expression of *Spp1* correlates with expression of other gonadotroph marker genes in a sex-specific manner in anterior pituitary tissue from developing rats. Correlation between *Spp1* vs. *Lhb* **(A,D)**, *Fshb* **(B,E)**, and *Cga* **(C,F)** expression. Data points for *Lhb*, *Fshb*, and *Cga* are derived from (35). n.s., non-significant. Gray circles, prepubertal females; white circles, postpubertal females. **(G)** The expression of gonadotroph marker genes and *Spp1* in postpubertal females during estrous cycle: P, proestrus; E, estrus; D, diestrus-1 (metestrus) + diestrus-2.

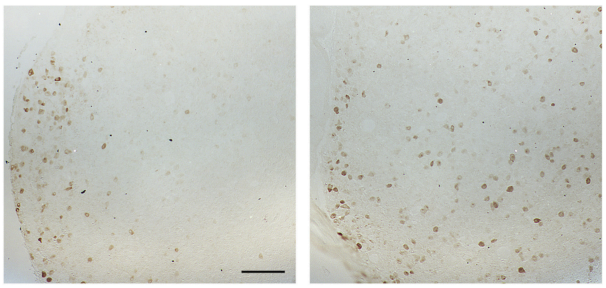

FIGURE 3 | The sex-specific expression pattern of osteopontin (OPN) in the anterior pituitary tissue from prepubertal female and male rats. Male tissue sections contained greater number of labeled cells which were more homogenously distributed, when compared to female pituitary tissue. Scale bar of 200 μm applies to both images.

vs. proestrus) and the largest was during the diestrus (17.36 ± 1.89, $n = 15$; $P < 0.01$ vs. proestrus).

In contrast to gonadotroph marker genes, no correlation was observed between *Spp1* expression vs. expression of *Pomc*, a marker gene for corticotrophs and melanotrophs, *Tshb*, a marker gene for thyrotrophs, *Gh1*, a marker gene for somatotrophs, and *Prl*, a marker gene for lactotrophs (data not shown).

These results suggest that *Spp1* is expressed in pituitary gland in a sex-specific manner and that expression of this gene during development is synchronized with expression of gonadotroph signature genes, a finding consistent with a hypothesis that this gene is active only in gonadotrophs. However, the expression of *Spp1* is regulated differently than the expression of gonadotroph marker genes during the estrous cycle.

In vivo OPN Is Specifically Expressed in Pituitary Gonadotrophs

To clarify this hypothesis, we performed an immunohistochemical analysis of pituitary tissue using antibodies specific for OPN, a protein encoded by *Spp1*, LHβ, and FSHβ. This analysis confirmed that OPN was also present in both female and male pituitary cells during sexual maturation. **Figure 3** shows OPN-positive cells in female and male pituitaries from prepubertal animals, with more positive cells observed in male pituitaries. In parallel to mRNA expression, OPN-positive cells were also visible in peripubertal and postpubertal anterior pituitaries from both sexes and with greater number of labeled cells in male tissue sections (data not shown). Finally, double immunohistochemical labeling indicated that all OPN-positive cells in males and females were also LHβ positive, i.e., that *Spp1*/OPN are specifically expressed in LHβ-positive gonadotrophs (**Figure 4**). Finally, triple immunohistochemical labeling indicated that most of the OPN positive cells in males were positive for both LHβ and FSHβ (**Figure 5**).

However, OPN labeling was not visible in all gonadotrophs (**Figures 4**, **5**), suggesting that *in vivo* expression of OPN was bellow detection by immunohistochemistry in a fraction of these cells. In parallel to *Spp1* expression during estrous cycle (**Figure 2G**), greater number of OPN-positive gonadotrophs could be observed in diestrus when compared to other stages of the estrous cycle. Furthermore, OPN-positive gonadotrophs were more intensely labeled in diestrus (**Figure 6**). Finally, the difference in the expression of gonadotroph marker genes and *Spp1* during estrous cycle suggest that *Spp1* expression was probably independent of the status of GnRH secretion.

Basal *Spp1* Expression Is Upregulated in Cultured Pituitary Cells

Next, we investigated the expression of basal *Spp1* in primary pituitary cell cultures. After cell dispersion, the *Spp1* expression progressively increased as a function of time in both female and male pituitary cell cultures. The upregulation of *Spp1* expression persisted over several days; **Figure 7A** illustrates the time course of upregulation of *Spp1* expression during the first 3 days of culturing in medium 199 containing 10% horse serum. Similar growth profiles in *Spp1* expression were observed in cells cultured in medium 199 containing fetal calf serum (data not shown). In cells cultured in serum-free and 0.1% BSA-containing medium, the growth in gene expression was not abolished and the rate of expression was only slightly attenuated. For example, in cells from 7-week old females cultured overnight in horse-serum containing medium or 0.1% BSA-containing medium, the expression of *Spp1* was 53.21 ± 2.52 and 43.88 ± 3.26, respectively. In contrast to *Spp1*, the expression of *Gnrhr* decreased progressively with culturing time [**Figure 7B** and (38)]. This indicates that loss of pulsatile GnRH stimulation and tridimensional pituitary structure has opposite effect on expression of *Spp1* and gonadotroph marker genes.

The *Spp1* expression in pituitary cells cultured in poly-D-lysine coated wells for 48 h was 112 ± 24, while in collagen-coated wells was 134 ± 36, both relative to *Gapdh* expression ($n = 6$). However, the rate in *Spp1* expression decreased in female pituitary cells cultured in horse serum- and BSA-containing medium when media were replaced with fresh medium once or twice during 72 h incubation (**Figure 7C**). In contrast, the decay in *Gnrhr* expression was not affected by washing procedure (**Figure 7D**). These observations are consistent with a hypothesis that an autocrine or paracrine factor, other than GnRH (39), stimulates *Spp1* expression.

Spp1 mRNA Expression Is Not Regulated by GnRH

To evaluate effects of GnRH on *Spp1* mRNA expression more directly, we performed two types of experiments, *in vitro* and *in vivo*. **Figure 8** summarizes experiments done with pituitary cells derived from 4-week old females and males. Treatment of 20 h-old cultures of these cells with 10 nM GnRH during 8 h incubation did not affect *Spp1* expression (**Figure 8A**). In contrast, the expression of a sister gene *Dmp1* increased in a time-dependent manner, with a peak in response after 4 h of incubation (**Figure 8B**). The expression of *Gnrhr* was also stimulated by GnRH in both pituitary cultures, from females and males (**Figure 8C**).

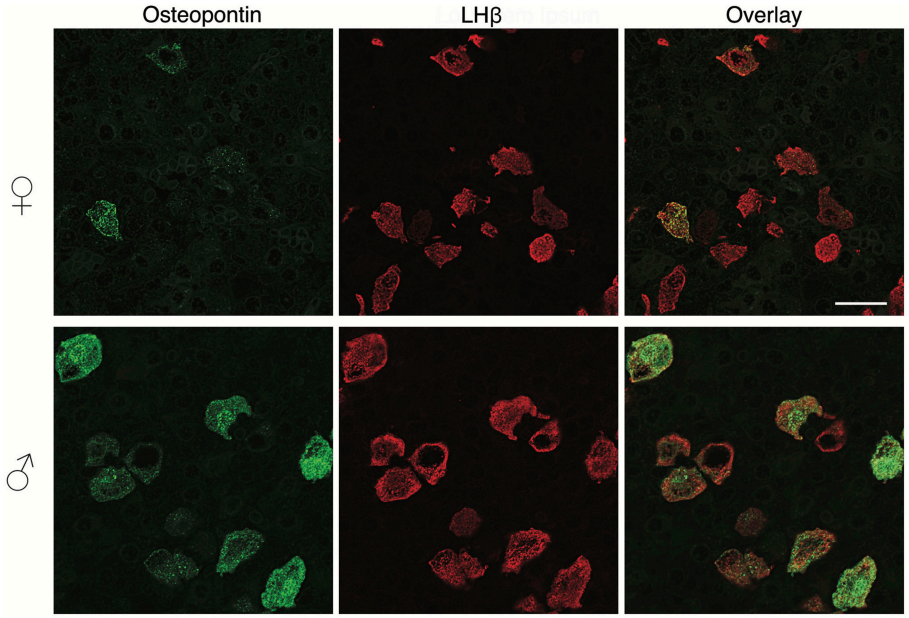

FIGURE 4 | OPN expression in the adult pituitary tissue is sex-specific and restricted to gonadotrophs. Immunofluorescence staining for OPN and LHβ in the anterior pituitary tissue from adult female (top) and male (bottom) rats. Scale bar of 20 μm applies to all images.

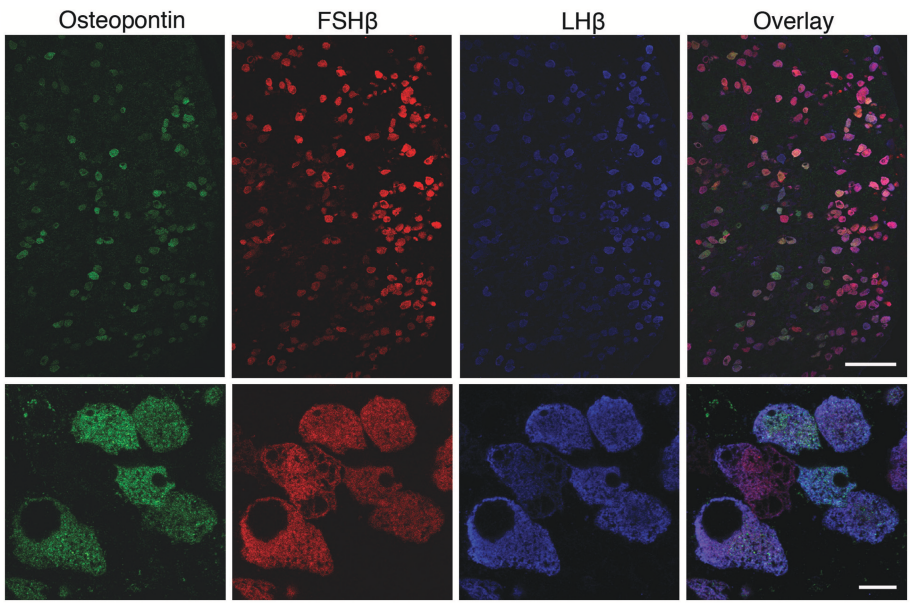

FIGURE 5 | Triple immunofluorescence labeling for OPN (green), FSHβ (red), and LHβ (blue) in adult male rat pituitary tissue. Low magnification images (upper row) show that all OPN-positive cells were also LHβ-positive; most of the OPN-positive cells are both FSHβ and LHβ-positive. However, OPN was not visible in all gonadotrophs. Scale bar: 100 μm. High -magnification of triple immunofluorescence labeling for OPN, FSHβ and LHβ (bottom row) shows a cluster of gonadotroph cells, most of which show immunoreactivity for all three markers. Scale bar: 10 μm.

The results of *in vivo* experiments with 4-week-old female and male rats were summarized in **Figure 9**. Animals were intraperitoneally injected with saline solution (solvent) or 5 μg of buserelin acetate, a GnRHR agonist. Animals were sacrificed 3, 6, and 9 h after injection, blood was collected for serum LH measurements and pituitary glands were removed for qRT-PCR analysis. LH measurements confirmed that stimulus secretion coupling was operative under these experimental conditions (data not shown). We also observed a progressive *Dmp1* expression of comparable levels to those observed in cultured pituitary cells (**Figure 9A**). However, buserelin acetate treatment did not affect *Spp1* expression (**Figure 9B**).

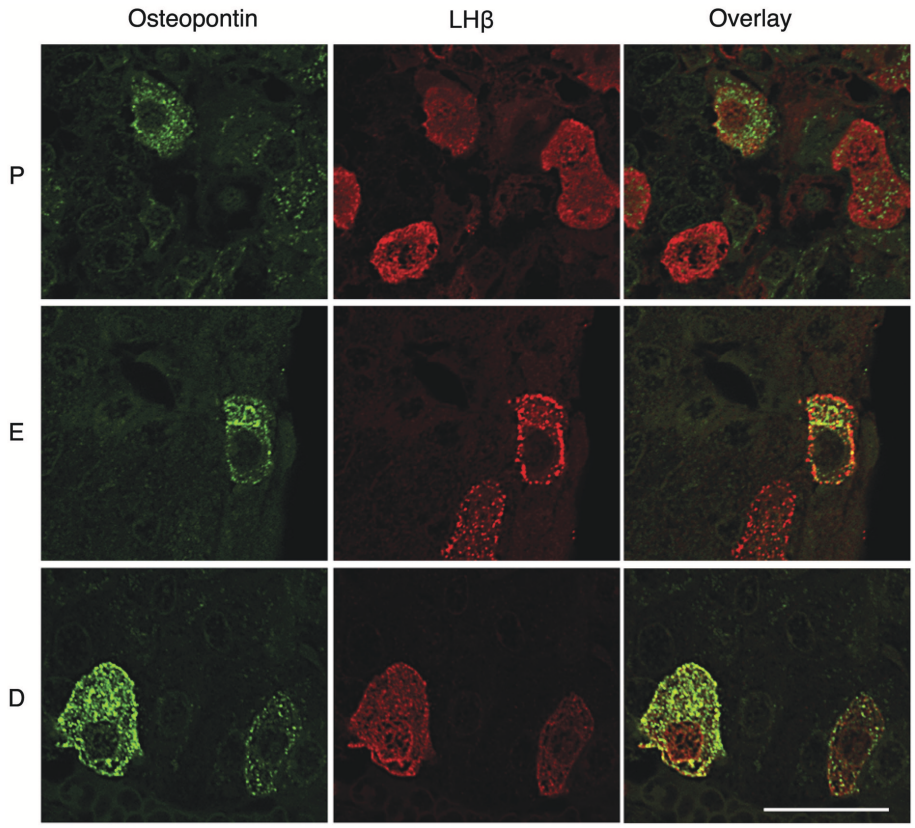

FIGURE 6 | Estrous cycle influences OPN expression in the pituitary tissue. Gonadotrophs were more intensively labeled in diestrus (D), when compared to proestrus (P) and estrus (E). Scale bar = 20 μm applies to all images.

We also performed two *in vitro* experiments using pituitary cells from 7-week-old female rats. In the first experiment, cells were cultured overnight in GnRH-free medium, and after that for 2–60 h in the presence and absence of 10 nM GnRH. Under these conditions, GnRH-induced expression of *Dmp1* (**Figure 10A**, top), with kinetics comparable to that we reported earlier, with a peak in response observed after 6 h GnRH application, followed by a decay to levels that were on the edge of detection by qRT-PCR (32). The subsequent application of GnRH was inefficient, indicating that decay in *Dmp1* expression is not due to degradation of GnRH but reflects desensitization of response (data not shown). In contrast, during 60 h of incubation, there was a progressive increase in *Spp1* expression in both controls and 10 nM GnRH-treated cells, but no significant differences between treated and untreated cells at the same time points (**Figure 10A**, bottom).

In the second experiment, pituitary cells were initially cultured for 96 h without GnRH, followed by washing and addition of fresh medium supplemented with 10 nM GnRH. Under these conditions, we also observed a time-dependent induction of *Dmp1* expression by GnRH (**Figure 10B**, top), indicating that GnRH-induced intracellular signaling and stimulus transcriptional coupling were still operative. However, the expression of *Spp1* was comparable in all groups (**Figure 10B**, bottom). *In vivo* injected buserelin acetate to 7-week old females also stimulated *Dmp1* expression and did not affect *Spp1* expression (**Figure 10C**).

In vitro experiments were also done with pituitary cultures from 2-, 3-, 6-, 8-, 9-, and 12-week old animals and no change in *Spp1* expression was observed in GnRH (10 nM)-treated cells (data not shown), further supporting the view that GnRH does not regulate *Spp1* expression. We also treated cultured cells with 100 nM thyrotropin-releasing hormone, 100 nM corticotropin-releasing hormone, 100 nM somatostatin-28, 1 μM dopamine, 1 μM oxytocin, 1 μM PACAP28, 1 μM endothelin-1, 25 ng/ml activin, 100 ng/ml IGF, 10 ng/ml EGF, and 2 ng//ml TGFβ1 for 6 h. None of these treatments affected *Spp1* expression *in vitro* (data not shown).

DISCUSSION

The expression of OPN mRNA in pituitary gonadotrophs has been reported previously (33). Here we provide further evidence that *Spp1* is expressed in gonadotrophs but not in other pituitary cell types. First, the developmental pattern of *Spp1*/OPN is sex-specific and comparable to that observed for *Lhb*, *Fshb*, and *Gnrhr* (35), the well-established marker genes for gonadotrophs (40). Second, there was no correlation between *Spp1* vs. *Gh1, Prl, Tshb*, and *Pomc*, the marker genes for somatotrophs, lactotrophs,

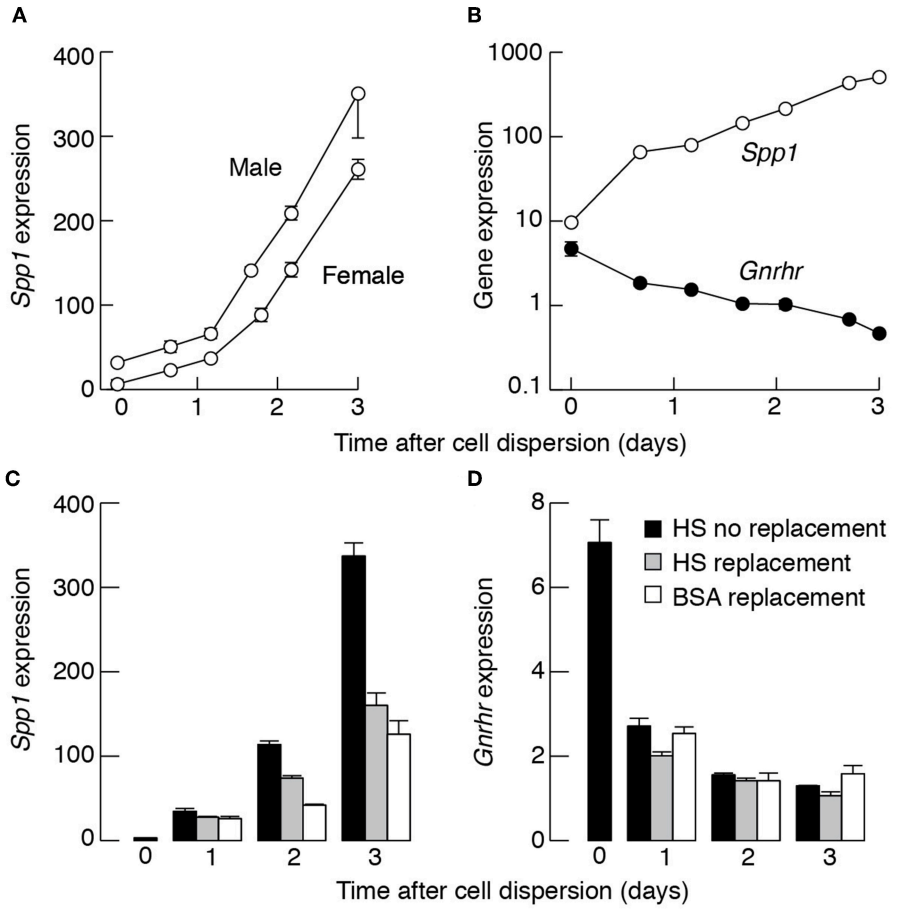

FIGURE 7 | Culturing of dispersed pituitary cells stimulates *Spp1* expression. **(A)** The time course of basal *Spp1* expression in cultured pituitary cells. Cells were derived from 7-week-old female and male rats. Zero indicates *Spp1* expression levels immediately after cell dispersion. Cells were cultured in medium 199 containing horse serum. **(B)** Opposite effects of dispersion and culturing of pituitary cells from females on *Spp1* and *Gnrhr* expression. In **(A)** and **(B)**, cells were continuously cultured in medium 199 containing horse serum without replacement of old medium with fresh. **(C,D)** Effects of replacement of culturing medium on rate of basal *Spp1* **(C)** and *Gnrhr* **(D)** expression. Old media were replaced with fresh media after 12, 36, and 60 h incubation and cells were collected for mRNA extraction immediately after dispersion, and 24, 48, and 72 h after dispersion.

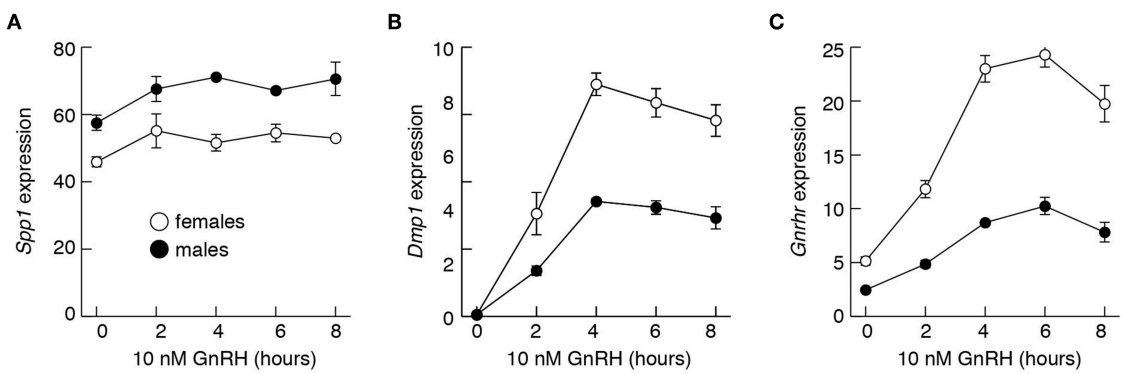

FIGURE 8 | The lack of an ability of GnRH to stimulate *Spp1* expression in cultured anterior pituitary cells. Experiments were performed with cells from 4-week-old females and males. Cells were cultured overnight in medium 199 containing horse serum, which was replaced with BSA-containing medium 199 supplemented with 10 nM GnRH in the morning, cultured for 0 to 8 h, and mRNA was extracted for measurement of *Spp1* **(A)**, *Dmp1* **(B)**, and *Gnrhr* **(C)** expression.

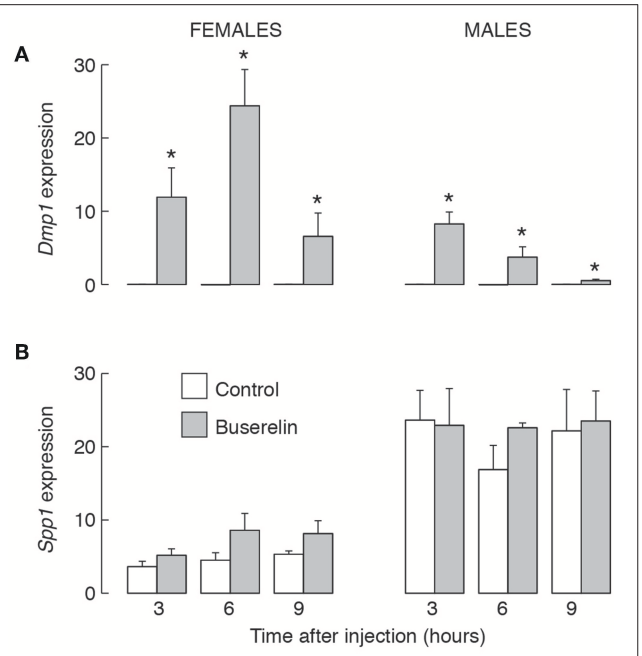

FIGURE 9 | The lack of an effect of GnRHR agonist injection *in vivo* on *Spp1* mRNA expression in anterior pituitary gland. Prepubertal (4-week-old) female and male rats were injected with buserelin acetate, a GnRHR agonist (5 μg/0.4 ml per animal or solvent (0.4 ml PBS) intraperitoneally. Three, 6 or 9 h after injection, anterior pituitary glands were removed and qRT-PCR analysis for *Dmp1* **(A)**, *Spp1* **(B)**, and *Gapdh* was performed; asterisks indicate significant differences vs. the corresponding solvent injected animals; $p < 0.05$ or higher.

thyrotrophs, and corticotrophs, respectively. Third, immunohistochemical analysis showed the expression of OPN only in LHβ- and LHβ/FSHβ- positive cells. Our recent single cell RNA-sequence also showed the expression of *Spp1* in gonadotrophs only (NCBI Sequence Read Archive accession SRP151788). Thus, *Spp1* is an additional gonadotroph-specific gene.

The expression of gonadotroph-marker genes, such as *Lhb*, *Fshb*, and *Gnrhr,* is regulated by GnRH (41). Previously we also showed that pulsatile GnRH application facilitated expression of numerous genes, including *Fshb*, *Cga*, and *Gnrhr*, but not *Spp1* (32). Here we show that both *in vitro* and *in vivo* activations of GnRHR were ineffective in induction of *Spp1*. We also show that *Spp1* expression varies during estrous cycle, with the pattern not comparable to endogenous GnRH release. Activation of other G protein-coupled receptors expressed in gonadotrophs was also ineffective. In other tissues, the expression of OPN is up-regulated by numerous growth and differentiation factors, including transforming growth factor-β superfamily, bone morphogenic proteins, epidermal growth factors, platelet-derived growth factor, and inflammatory cytokines. Also, steroids, retinoic acid, glucocorticosteroids, and 1.25-dihydroxyvitamin D3 increase OPN expression (42). In our experiments, IGF, EGF, TGFβ1, and activin were ineffective.

We established previously that *Dmp1* is a gonadotroph-specific gene within cells of anterior pituitary gland (32). However, the expression of these two SIBLING genes in gonadotrophs varies. Both genes are expressed *in vivo* in a sex-specific manner, but the expression of *Spp1* was always higher in males and *Dmp1* was always better expressed in females. In postpubertal females, the expression of *Spp1* was largest during the diestrus stage of estrous cycle, whereas the expression of *Dmp1* was most prominent during the late proestrus. In further contrast to *Spp1*, the expression of *Dmp1 in vivo* and *in vitro* was regulated by GnRHR; continuous GnRH application caused a transient stimulation of *Dmp1* expression followed by prolonged desensitization. Thus, although *Dmp1* is a sister gene of *Spp1*, it follows the expression pattern of *Lhb*, *Fshb*, and *Gnrhr* (32, 41).

The role of pituitary gonadotrophs in reproduction is well-established. Work with expression of SIBLINGs in pituitary gland indicates that gonadotrophs may have an additional cell-type specific function in anterior pituitary gland. At the present time, this function is unknown. Based on functions of OPN and DMP1 in other tissues, we may speculate that these proteins contribute to the proper organization of the cell-ECM tridimensional network, the former in GnRHR-dependent manner and the latter in a GnRHR-independent manner. The work on development of pituitary gland has indicated that, at birth, the pituitary cell types are roughly organized into layers with gonadotrophs being the most ventral (43). However, by adulthood spatial organization of the cell types appears more random (44). It has also been proposed that layering of pituitary cell types at birth could be required to establish networks of specific cell types, rather than a relationship with the timing of cell cycle exit (45). Thus, it is reasonable to speculate the potential role of these proteins in postnatal organization of pituitary.

We also present evidence, for the first time, that *Spp1* expression increased progressively after pituitary cell dispersion in both female and male cultures, reaching 30–40-fold increase in mRNA levels within 3 days. Such response suggests that OPN signals to other pituitary cell types for changes in cell-matrix network structure. *Spp1* is also upregulated as early as 6 h after skin wounding and healing was altered in mice lacking a functional *Spp1*. This and some additional analyses led the authors to conclude that OPN has a role in tissue remodeling and during matrix reorganization after injury (46). In general, OPN has been shown to promote attachment and spreading of a variety of cell types through its glycine-arginine-glycine-aspartate-serine cell binding domain, i.e., OPN can be classified as an adhesive protein (47). Thus, OPN may represent an initial signal for reconstruction of tridimensional structure of pituitary gland.

Consistent with this hypothesis, it has been shown that rat anterior pituitary cells *in vitro* can partly reconstruct the topographic nature of the pituitary gland, which includes few junctional complexes between hormone-producing cells (48). More recently, the same group reported about reassembly of anterior pituitary organization by hanging drop cell culture. Specifically, the authors reported that the topographic affinities of hormone-producing cells were maintained, that folliculostellate cells were interconnected with typical cytoplasmic protrusions to form tridimensional network, with the major ECM components, collagens and laminin, being deposited and distributed around the cells (49). It has also been reported that gonadotrophs can signal to the lactotrophs through the release of a paracrine

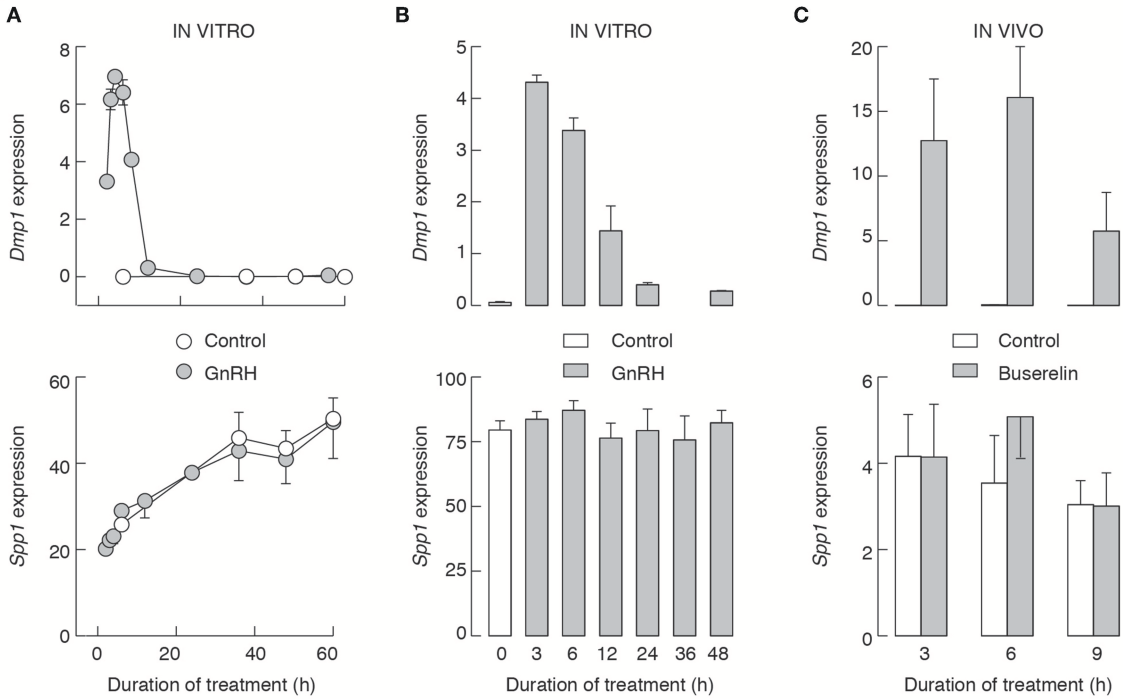

FIGURE 10 | The lack of an effect of GnRHR activation on *Spp1* expression *in vitro* **(A,B)** and *in vivo* **(C)**. Experiments were performed with 7-week-old females. **(A)** Pituitary cells were cultured overnight in medium 199 containing horse serum, washed in the morning, and cultured in medium 199 supplemented with BSA with or without 10 nM GnRH for 0–60 h prior to the end of experiments, and samples were analyzed for *Dmp1* (top) and *Spp1* (bottom) mRNA expression. **(B)** 96 h old cell cultures were treated as described in **(A)**, up to 48 h, and samples were analyzed for *Dmp1* (top) and *Spp1* (bottom) mRNA expression. **(C)** Animals were injected with buserelin acetate or solvent as described above. Gray bars and circles, GnRH/buserelin acetate-treated cells/animals; white bars and circles, solvent-treated cells/animals.

humoral factor distinct from LH and FSH and in a GnRH-independent manner (50). Further studies indicated that a common alpha subunit of pituitary gonadotropins accounts for influence of gonadotrophs on lactotroph functions (51–53). We may speculate that OPN is another protein released by gonadotrophs, which contributes to the crosstalk among anterior pituitary cells.

Dispersion of pituitary cells could be considered as the stress situation for pituitary tissue. In general, OPN plays a role in immune regulation and stress responses (54). It has also a role in mediating oxidative stress (55), mechanical stress (56), and cellular stress (57). OPN also plays a significant role in the regulation of the hypothalamus-pituitary-adrenal axis hormones in animals exposed to chronic restraint stress (58). Cancer also reflects the loss of tissue organization and aberrant behavior of the cellular components and tumors have been likened to wounds that fail to heal (4). Not surprising, elevated OPN expression has been detected in numerous tumors (59–61). Elevated OPN levels were also detected in silent corticotroph adenomas and non-functioning gonadotroph adenomas (62). OPN expression is inhibitable on the levels of gene transcription and the RNA message, and its protein ligand activity can be blocked with antibodies or synthetic peptides, which led to idea to consider OPN as a candidate target for cancer therapy (63).

For understanding the signaling function of OPN in intact pituitary gland, dispersed pituitary cells, and pituitary tumors, it is critical to identify OPN receptors and cell types expressing these receptors. In general, OPN binds to several integrins, including αv(β1, β3, or β5), and (α4, α5, α8, or α9)β1, and is a ligand for CD44 receptor splice variants, specifically v6 and/or v7 possibly in conjunction with a β1 integrin (63). It is also known that exogenous addition of OPN to OPN-/- osteoclasts increased the surface expression of CD44 (64). The expression of αvβ3 integrin was reported in immortalized GH_3 lacto-somatotrophs (65). It has also been reported that cultured rat anterior pituitary cells expressed the β1 integrin subunit (11) as well as that integrin β1 signaling is required for the proliferation of folliculostellate cells in rat anterior pituitary gland under the influence of ECM (66). To our best knowledge, at the present time no data exist describing the expression of *Cd44* and its protein in normal mammalian anterior pituitary cells.

In summary, here we provide further evidence for the expression of *Spp1* and *Dmp1* in pituitary gonadotrophs, but not other pituitary cell types, in an age-, sex-, and estrous cycle stage-specific manner. Two genes also differ in regulation of their expression; *Dmp1* expression is regulated by GnRH, whereas *Spp1* expression increases progressively in culturing pituitary cells in a GnRH-independent manner, presumably in response to an unidentified paracrine factor. Further work should be focused on secretion of these two proteins by gonadotrophs under different experimental paradigms, characterization of integrin and CD44 receptors within the secretory and non-secretory anterior pituitary cells and their signaling pathways, and function in pituitary gland.

ETHICS STATEMENT

All experiments were approved by the NICHD Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SS and IB: conceptualization. IB, MJ, RP, DA, and MK: experimental work. SS: writing—original draft. SS, IB, MJ, and RP: writing—review and editing. SS and IB: data analysis and figure preparation, supervision, and funding acquisition.

FUNDING

This work was supported by the National Institute of Child Health and Human Development Intramural Program-Project ZIA HD 000195-24 (1) and Ministry of Education, Science, and Technological Development of the Republic of Serbia, Grant III 41014 (2).

REFERENCES

1. Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol*. (1998) 8:437–41. doi: 10.1016/S0962-8924(98)01362-2
2. Paez-Pereda M, Kuchenbauer F, Arzt E, Stalla GK. Regulation of pituitary hormones and cell proliferation by components of the extracellular matrix. *Braz J Med Biol Res*. (2005) 38:1487–94. doi: 10.1590/S0100-879X2005001000005
3. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. *Adv Drug Deliv Rev*. (2016) 97:4–27. doi: 10.1016/j.addr.2015.11.001
4. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. (2010) 123:4195–200. doi: 10.1242/jcs.023820
5. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. (2002) 110:673–87. doi: 10.1016/S0092-8674(02)00971-6
6. Fujiwara K, Jindatip D, Kikuchi M, Yashiro T. *In situ* hybridization reveals that type I and III collagens are produced by pericytes in the anterior pituitary gland of rats. *Cell Tissue Res*. (2010) 342:491–5. doi: 10.1007/s00441-010-1078-1
7. Horiguchi K, Kouki T, Fujiwara K, Kikuchi M, Yashiro T. The extracellular matrix component laminin promotes gap junction formation in the rat anterior pituitary gland. *J Endocrinol*. (2011) 208:225–32.
8. Horiguchi K, Syaidah R, Fujiwara K, Tsukada T, Ramadhani D, Jindatip D, et al. Expression of small leucine-rich proteoglycans in rat anterior pituitary gland. *Cell Tissue Res*. (2013) 351:207–12. doi: 10.1007/s00441-012-1513-6
9. Ramadhani D, Tsukada T, Fujiwara K, Azuma M, Kikuchi M, Yashiro T. Changes in laminin chain expression in pre- and postnatal rat pituitary gland. *Acta Histochem Cytochem*. (2014) 47:231–7. doi: 10.1267/ahc.14031
10. Tsukada T, Fujiwara K, Horiguchi K, Azuma M, Ramadhani D, Tofrizal A, et al. Folliculostellate cells are required for laminin release from gonadotrophs in rat anterior pituitary. *Acta Histochem Cytochem*. (2014) 47:239–45. doi: 10.1267/ahc.14036
11. Horacek MJ, Kawaguchi T, Terracio L. Adult adenohypophysial cells express beta 1 integrins and prefer laminin during cell-substratum adhesion. *In Vitro Cell Dev Biol Anim*. (1994) 30A:35–40. doi: 10.1007/BF02631416
12. Horacek MJ, Thompson JC, Dada MO, Terracio L. The extracellular matrix components laminin, fibronectin, and collagen IV are present among the epithelial cells forming Rathke's pouch. *Acta Anat*. (1993) 147:69–74. doi: 10.1159/000147484
13. Gonzalez B, Solano-Agama Mdel C, Gonzalez Del Pliego M, Mendoza-Garrido ME. Differences in cell migration of cultured pituitary cells from infantile and adult rats: participation of the extracellular matrix and epidermal growth factor. *Int J Dev Neurosci*. (2004) 22:231–9. doi: 10.1016/j.ijdevneu.2004.02.003
14. Denduchis B, Rettori V, McCann SM. Role of laminin on prolactin and gonadotrophin release from anterior pituitaries of male rats. *Life Sci*. (1994) 55:1757–65. doi: 10.1016/0024-3205(94)00344-0
15. Diaz ES, Rettori V, Suescun MO, Lustig L, McCann SM, Denduchis B. Role of type IV collagen in prolactin release from anterior pituitaries of male rats. *Endocrine*. (2002) 18:185–9. doi: 10.1385/ENDO:18:2:185
16. Kuchenbauer F, Hopfner U, Stalla J, Arzt E, Stalla GK, Paez-Pereda M. Extracellular matrix components regulate ACTH production and proliferation in corticotroph tumor cells. *Mol Cell Endocrinol*. (2001) 175:141–8. doi: 10.1016/S0303-7207(01)00390-2
17. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connect Tissue Res*. (2003) 44(Suppl. 1):33–40. doi: 10.1080/03008200390152061
18. Bellahcene A, Castronovo V, Ogbureke KU, Fisher LW, Fedarko NS. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nat Rev Cancer*. (2008) 8:212–26. doi: 10.1038/nrc2345
19. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Crit Rev Oral Biol Med*. (2004) 15:126–36. doi: 10.1177/154411130401500302
20. Kazanecki CC, Uzwiak DJ, Denhardt DT. Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. *J Cell Biochem*. (2007) 102:912–24. doi: 10.1002/jcb.21558
21. Staines KA, MacRae VE, Farquharson C. The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling. *J Endocrinol*. (2012) 214:241–55. doi: 10.1530/JOE-12-0143
22. Ogbureke KU, Fisher LW. Expression of SIBLINGs and their partner MMPs in salivary glands. *J Dent Res*. (2004) 83:664–70. doi: 10.1177/154405910408300902
23. Ogbureke KU, Fisher LW. Renal expression of SIBLING proteins and their partner matrix metalloproteinases (MMPs). *Kidney Int*. (2005) 68:155–66. doi: 10.1111/j.1523-1755.2005.00389.x
24. Yu H, Liu X, Zhong Y. The effect of osteopontin on microglia. *Biomed Res Int*. (2017) 2017:1879437. doi: 10.1155/2017/1879437
25. Wung JK, Perry G, Kowalski A, Harris PL, Bishop GM, Trivedi MA, et al. Increased expression of the remodeling- and tumorigenic-associated factor osteopontin in pyramidal neurons of the Alzheimer's disease brain. *Curr Alzheimer Res*. (2007) 4:67–72. doi: 10.2174/156720507779939869
26. Iczkiewicz J, Rose S, Jenner P. Osteopontin (Eta-1) is present in the rat basal ganglia. *Brain Res Mol Brain Res*. (2004) 132:64–72. doi: 10.1016/j.molbrainres.2004.09.013
27. Chabas D, Baranzini SE, Mitchell D, Bernard CC, Rittling SR, Denhardt DT, et al. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science*. (2001) 294:1731–5. doi: 10.1126/science.1062960
28. Comabella M, Pericot I, Goertsches R, Nos C, Castillo M, Blas Navarro J, et al. Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol*. (2005) 158:231–9. doi: 10.1016/j.jneuroim.2004.09.004
29. Terasawa M, Shimokawa R, Terashima T, Ohya K, Takagi Y, Shimokawa H. Expression of dentin matrix protein 1 (DMP1) in nonmineralized tissues. *J Bone Miner Metab*. (2004) 22:430–8. doi: 10.1007/s00774-004-0504-4
30. Fisher LW, Jain A, Tayback M, Fedarko NS. Small integrin binding ligand N-linked glycoprotein gene family expression in different cancers. *Clin Cancer Res*. (2004) 10:8501–11. doi: 10.1158/1078-0432.CCR-04-1072
31. Wei R, J.Wong PC, Kwok HF. Osteopontin – a promising biomarker for cancer therapy. *J Cancer*. (2017) 8:2173–83. doi: 10.7150/jca.20480
32. Kucka M, Bjelobaba I, Clokie SJ, Klein DC, Stojilkovic SS. Female-specific induction of rat pituitary dentin matrix protein-1 by GnRH. *Mol Endocrinol*. (2013) 27:1840–55. doi: 10.1210/me.2013-1068
33. Ehrchen J, Heuer H, Sigmund R, Schafer MK, Bauer K. Expression and regulation of osteopontin and connective tissue growth factor

transcripts in rat anterior pituitary. *J Endocrinol*. (2001) 169:87–96. doi: 10.1677/joe.0.1690087
34. Bargi-Souza P, Kucka M, Bjelobaba I, Tomic M, Janjic MM, Nunes MT, et al. Loss of basal and TRH-stimulated Tshb expression in dispersed pituitary cells. *Endocrinology*. (2015) 156:242–54. doi: 10.1210/en.2014-1281
35. Bjelobaba I, Janjic MM, Kucka M, Stojilkovic SS. Cell type-specific sexual dimorphism in rat pituitary gene expression during maturation. *Biol Reprod*. (2015) 93:21. doi: 10.1095/biolreprod.115.129320
36. Li S, Bjelobaba I, Yan Z, Kucka M, Tomic M, Stojilkovic SS. Expression and roles of pannexins in ATP release in the pituitary gland. *Endocrinology*. (2011) 152:2342–52. doi: 10.1210/en.2010-1216
37. Zemkova H, Kucka M, Bjelobaba I, Tomic M, Stojilkovic SS. Multiple cholinergic signaling pathways in pituitary gonadotrophs. *Endocrinology*. (2013) 154:421–33. doi: 10.1210/en.2012-1554
38. Bjelobaba I, Janjic MM, Tavcar JS, Kucka M, Tomic M, Stojilkovic SS. The relationship between basal and regulated Gnrhr expression in rodent pituitary gonadotrophs. *Mol Cell Endocrinol*. (2016) 437:302–11. doi: 10.1016/j.mce.2016.08.040
39. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Tomic M, Stojilkovic SS, et al. Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. *Endocrinology*. (2000) 141:1187–95. doi: 10.1210/endo.141.3.7392
40. McArdle CA, Roberson MS. *Gonadotropes and Gonadotropin-Releasing Hormone Signaling*. Sasn Diego, CA: Elsevier (2015). doi: 10.1016/B978-0-12-397175-3.00010-7
41. Ferris HA, Shupnik MA. Mechanisms for pulsatile regulation of the gonadotropin subunit genes by GNRH1. *Biol Reprod*. (2006) 74:993–8. doi: 10.1095/biolreprod.105.049049
42. Sodek J, Ganss B, McKee MD. Osteopontin. *Crit Rev Oral Biol Med*. (2000) 11:279–303. doi: 10.1177/10454411000110030101
43. Davis SW, Mortensen AH, Camper SA. Birthdating studies reshape models for pituitary gland cell specification. *Dev Biol*. (2011) 352:215–27. doi: 10.1016/j.ydbio.2011.01.010
44. Stallings CE, Kapali J, Ellsworth BS. Mouse models of gonadotrope development. *Prog Mol Biol Transl Sci*. (2016) 143:1–48. doi: 10.1016/bs.pmbts.2016.08.001
45. Bonnefont X, Lacampagne A, Sanchez-Hormigo A, Fino E, Creff A, Mathieu MN, et al. Revealing the large-scale network organization of growth hormone-secreting cells. *Proc Natl Acad Sci USA*. (2005) 102:16880–5. doi: 10.1073/pnas.0508202102
46. Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BL. Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest*. (1998) 101:1468–78. doi: 10.1172/JCI2131
47. Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, et al. Osteopontin expression and distribution in human carcinomas. *Am J Pathol*. (1994) 145:610–23.
48. Noda T, Kikuchi M, Kaidzu S, Yashiro T. Rat anterior pituitary cells *in vitro* can partly reconstruct *in vivo* topographic affinities. *Anat Rec A Discov Mol Cell Evol Biol*. (2003) 272:548–55. doi: 10.1002/ar.a.10065
49. Tsukada T, Kouki T, Fujiwara K, Ramadhani D, Horiguchi K, Kikuchi M, et al. Reassembly of anterior pituitary organization by hanging drop three-dimensional cell culture. *Acta Histochem Cytochem*. (2013) 46:121–7. doi: 10.1267/ahc.13015
50. Denef C, Andries M. Evidence for paracrine interaction between gonadotrophs and lactotrophs in pituitary cell aggregates. *Endocrinology*. (1983) 112:813–22. doi: 10.1210/endo-112-3-813
51. Begeot M, Hemming FJ, Dubois PM, Combarnous Y, Dubois MP, Aubert ML. Induction of pituitary lactotrope differentiation by luteinizing hormone alpha subunit. *Science*. (1984) 226:566–8. doi: 10.1126/science.6208610
52. Chabot V, Magallon T, Taragnat C, Combarnous Y. Two free isoforms of ovine glycoprotein hormone alpha-subunitstrongly differ in their ability to stimulate prolactin release from foetal pituitaries. *J Endocrinol*. (2000) 164:287–97. doi: 10.1677/joe.0.1640287
53. Denef C. Paracrinicity: the story of 30 years of cellular pituitary crosstalk. *J Neuroendocrinol*. (2008) 20:1–70. doi: 10.1111/j.1365-2826.2007.01616.x
54. Wang KX, Denhardt DT. Osteopontin: role in immune regulation and stress responses. *Cytokine Growth Factor Rev*. (2008) 19:333–45. doi: 10.1016/j.cytogfr.2008.08.001
55. Itoh Y, Yasui T, Okada A, Tozawa K, Hayashi Y, Kohri K. Examination of the anti-oxidative effect in renal tubular cells and apoptosis by oxidative stress. *Urol Res*. (2005) 33:261–6. doi: 10.1007/s00240-005-0465-7
56. Fujihara S, Yokozeki M, Oba Y, Higashibata Y, Nomura S, Moriyama K. Function and regulation of osteopontin in response to mechanical stress. *J Bone Miner Res*. (2006) 21:956–64. doi: 10.1359/jbmr.060315
57. Wai PY, Kuo PC. The role of Osteopontin in tumor metastasis. *J Surg Res*. (2004) 121:228–41. doi: 10.1016/j.jss.2004.03.028
58. Wang KX, Shi YF, Ron Y, Kazanecki CC, Denhardt DT. Plasma osteopontin modulates chronic restraint stress-induced thymus atrophy by regulating stress hormones: inhibition by an anti-osteopontin monoclonal antibody. *J Immunol*. (2009) 182:2485–91. doi: 10.4049/jimmunol.0803023
59. Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, et al. Osteopontin at the crossroads of inflammation and tumor progression. *Mediators Inflamm*. (2017) 2017:4049098. doi: 10.1155/2017/4049098
60. Shi L, Wang X. Role of osteopontin in lung cancer evolution and heterogeneity. *Semin Cell Dev Biol*. (2017) 64:40–7. doi: 10.1016/j.semcdb.2016.08.032
61. Briones-Orta MA, Avendano-Vazquez SE, Aparicio-Bautista DI, Coombes JD, Weber GF, Syn WK. Osteopontin splice variants and polymorphisms in cancer progression and prognosis. *Biochim Biophys Acta Rev Cancer*. (2017) 1868:93–108A. doi: 10.1016/j.bbcan.2017.02.005
62. Mete O, Hayhurst C, Alahmadi H, Monsalves E, Gucer H, Gentili F, et al. The role of mediators of cell invasiveness, motility, and migration in the pathogenesis of silent corticotroph adenomas. *Endocr Pathol*. (2013) 24:191–8. doi: 10.1007/s12022-013-9270-y
63. Weber GF. The metastasis gene osteopontin: a candidate target for cancer therapy. *Biochim Biophys Acta*. (2001) 1552:61–85.
64. Chellaiah MA, Kizer N, Biswas R, Alvarez U, Strauss-Schoenberger J, Rifas L, et al. Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. *Mol Biol Cell*. (2003) 14:173–89. doi: 10.1091/mbc.e02-06-0354
65. Kong D, Liu Y, Zuo R, Li J. DnBP-induced thyroid disrupting activities in GH3 cells via integrin alphavbeta3 and ERK1/2 activation. *Chemosphere*. (2018) 212:1058–66. doi: 10.1016/j.chemosphere.2018.09.007
66. Horiguchi K, Fujiwara K, Ilmiawati C, Kikuchi M, Tsukada T, Kouki T, et al. Caveolin 3-mediated integrin beta1 signaling is required for the proliferation of folliculostellate cells in rat anterior pituitary gland under the influence of extracellular matrix. *J Endocrinol*. (2011) 210:29–36. doi: 10.1530/JOE-11-0103

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 26 June 2019
doi: 10.3389/fendo.2019.00422

Magnocellular Vasopressin and the Mechanism of "Glucocorticoid Escape"

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It is now widely accepted that magnocellular vasopressinergic neurons in the supraoptic and paraventricular nuclei participate in the control of adrenocorticotropin secretion by the anterior pituitary gland. However, it remains to be explored in further detail, when and how these multifunctional neurons are involved in the control of anterior pituitary function. This paper highlights the role of magnocellular vasopressin in the hypothalamic pituitary adrenocortical axis with special reference to escape from glucocorticoid feedback inhibition. The signaling mechanisms underlying glucocorticoid escape by pituitary corticotrope cells, as well as the wider physiologic and pathologic contexts in which escape is known to occur—namely strenuous exercise, and autoimmune inflammation will be considered. It is proposed that by inducing escape from glucocorticoid feedback inhibition at the pituitary level, magnocellular vasopressin is critically important for the anti-inflammatory, and immunosuppressant actions of endogenous corticosteroids.

Keywords: vasopressin, adrenal corticosteroids, ACTH, dexamethasone non-suppression, interleukin-6, agonist-induced plasticity

OPEN ACCESS

Edited by:
László Hunyady, Semmelweis University, Hungary

Reviewed by:
Hana Zemkova, Institute of Physiology (ASCR), Czechia
Maristela Oliveira Poletini, Federal University of Minas Gerais, Brazil

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 March 2019*
Accepted: *12 June 2019*
Published: *26 June 2019*

Citation:
Antoni FA (2019) Magnocellular Vasopressin and the Mechanism of "Glucocorticoid Escape". Front. Endocrinol. 10:422. doi: 10.3389/fendo.2019.00422

INTRODUCTION

This paper is the consequence of a symposium held in the honor of the late Dr. Kevin J. Catt (1932–2017) at Semmelweis University in Budapest. As a first year student at this institution, which has a tradition of teaching medicine for 250 years, I was handed the course textbook, "Functional Anatomy" by János Szentágothai (Schimmer) (1). First published as a single author book in 1971, this tome managed a readable, if in places decidedly subjective synthesis of classic gross anatomy and histology with modern studies of ultrastructure, physiology, and biochemistry. The didactic approach was unashamedly teleologic in emphasizing the unity of form and function. However, it went beyond Aristotelian metaphysics. It was an almost poetic celebration of the harmony of the human body, of how, from single cells to complex organs, structure reflected purpose, and function.

The scientific oeuvre of Kevin Catt heralds the beginning of a new era where the structures and regulatory properties of cells are pared back to their ultimate molecular constituents. Now in full-swing, this era of discovery is best symbolized by the complex analysis of individual cells, involving the combination of multi-parameter imaging with transcriptomics, and proteomics (2). We are able to map how the seemingly stochastic expression of genes underpins the functional plasticity of cell populations to support macroscopic physiologic reflexes and control mechanisms. This review aims to follow the path of Szentágothai in the quest for achieving the synthesis of structure and function, also incorporating the molecular insights of cellular signaling pathways pioneered by Kevin Catt.

AN OUTLINE OF THE HYPOTHALAMIC-PITUITARY-ADRENOCORTICAL AXIS

The hypothalamic-pituitary-adrenocortical system comprises a classic neuroendocrine regulatory feedback circuit. Neuroendocrine neurons in the hypothalamus drive the secretion of adrenocorticotropin (ACTH) by releasing neuropeptides, namely 41-residue corticotropin releasing-factor (CRF) and vasopressin (VP), into hypophysial portal blood-vessels that irrigate the anterior pituitary gland (3). These neurons receive a large number of diverse neural inputs from various parts of the brain that contribute to the hormonal response to stress (4–8). The production and secretion of steroid hormones—glucocorticoids, as well as mineralocorticoids—by the adrenal cortex is stimulated by ACTH secreted by the pituitary gland. An important effect of the steroids is to provide inhibitory feedback at the level of hypothalamic neroendocrine neurons, adenohypophysial corticotropes, as well as the higher brain centers involved in the control of neuroendocrine neurons (9, 10). The relative importance of the site(s) of feedback inhibition that govern the size and duration of the HPA response to stress may be dependent on the biological paradigm analyzed. In clinical practice, HPA responsiveness is usually assessed by some form of the dexamethasone suppression-test (11), utilizing a synthetic steroid largely selective for Type II glucocorticoid receptors (10). The main site of feedback tested by this paradigm appears to be the anterior pituitary corticotrope cell (12, 13).

VASOPRESSIN

Vasopressin (VP) is a small, COOH-terminally amidated peptide chiefly synthesized by nerve cells, hence designated as a neuropeptide. Arginine-vasopressin is the most common form in mammals, but in some species Arg is replaced by Lys see review by Bichet (14). Vasopressin is elaborated upon the processing of a larger protein precursor—preprovasopressin (15), which is packaged and processed in dense-core secretory granules that are transported to sites of release along the axon as well as the dendrites (15, 16). Note that neurophysin II and copeptin, the COOH-terminal segment of the precursor, are also co-secreted with VP. The biological role of copeptin is unknown. However, its stability in human plasma and immunogenicity has been exploited to use copeptin as surrogate for the activity of VP-secreting neurons with intriguing results (17, 18). The cellular actions of VP are mediated by G protein-coupled 7-transmembrane domain receptors. Of these, the V1b isoform appears most relevant for the stress response (19, 20).

FUNCTIONAL NEUROANATOMY OF VASOPRESSINERGIC NEURONS OF THE HYPOTHALAMUS

The Magnocellular System

The magnocellular neurosecretory system of the hypothalamus was discovered a relatively long time ago, on the basis of the distinct histochemical reaction produced by the high amounts of neurohormone elaborated by these neurons reviewed by Scharrer (21). The cell bodies are found in the supraoptic and paraventricular hypothalamic nuclei and send their axons to terminate in the neurohemal interface zone located at the bottom of the hypothalamus. The neurohemal interface is comprised of the median eminence and the posterior lobe of the pituitary gland also referred to as the neurohypophysis. Magnocellular neurons constitute the classic antidiuretic system that is activated by hypernatremia or hypovolemia and releases VP into the systemic circulation (14). Subsequent work has shown that the axons of magnocellular neurons also release VP *en-passant* in the internal zone of the median eminence (22–24). The VP released by this process appears in pituitary portal blood that irrigates the anterior pituitary gland [(25), also see **Box 1**]. It is of note, that magnocellular VP neurons also release vasopressin from their dendrites (16). Whether or not dendritic release contributes to the control of the pituitary secretion of ACTH remains to be clarified. However, it is well-established that magnocellular neurons make various forms of contact with CRF producing cells in the medial parvicellular paraventricular nucleus of the hypothalamus (26, 27). Moreover, Plotsky and co-workers found that in rats VP given into the 3rd cerebral ventricle attenuated the release of CRF into hypophysial portal blood and, conversely, inhibition of AVP action increased the levels of CRF (28). These results indicate that there may be a functional link between the magnocellular VP neurons and parvicellular neurons producing CRF.

Parvicellular Neurons Producing CRF and VP

A second population of VP-ergic neurons projecting to the median eminence is located in the medial parvicellular part of the hypothalamic paraventricular nucleus. These are small neurons that also co-produce and co-package CRF with VP (29, 30) and are the key neuroendocrine neurons that regulate the pituitary secretion of ACTH (31, 32). With respect to the stress response two functional differences between the magnocellular and parvicellular neurons are of note. First, under

Box 1 | Six decades of dogma-busting: The magnocellular vasopressinergic neuron as a modulator of anterior pituitary ACTH secretion.

1. Neurosecretion into the bloodstream-non-conforming to neuron doctrine.
2. Negates single hypothalamic releasing hormone for each pituitary hormone hypothesis.
3. VP is not just the antidiuretic hormone (ADH).
4. Secretory path from internal zone of the median eminence into pituitary portal blood-"*en passant*" neuropeptide release.
5. Negates the hyophyseotrophic area = the mediobasal hypothalamus concept.
6. VP alone has no ACTH releasing effect at physiologic concentrations i.e., it is a new type of neuroendocrine modulator, a major action of which is to induce glucocorticoid resistance at the level of the corticotrope cell.

normal conditions, magnocellular neurons produce up to 10-fold more VP than parvicellular neurons (30, 33). Second, the production and release of VP by parvicellular neurons is under prominent inhibitory control by adrenal corticosteroids (34), while that in magnocellular neurons is not (35).This is despite the fact that magnocellular VP neurons express Type II glucocorticoid receptors (36). In the absence of adrenal corticosteroids, VP release from the parvicellular neurons is massively increased and becomes comparable to that from magnocellular neurons (33, 37).

The Relevance of Magno- and Parvi-Cellular VP-Ergic Neurons to the Stress Response

The role of VP and its pituitary V1b receptor in the stress response has been analyzed in considerable detail in various models, including genetically vasopressin-deficient Brattleboro rats (38) and V1b receptor gene-deleted mice (20). These studies, and many others see (39) for review, support an important role for VP in the stimulatory control of stress-induced ACTH secretion. However, very few studies have attempted to distinguish between the effects of parvicellular and magnocellular VP. Plotsky and Thivikraman first produced evidence showing that glucocorticoid negative feedback appeared to be absent in conscious rats subjected to repeated moderate hemorrhage (40). Subsequently, these workers showed that HPA activation by air-puff startle, a psychological stressor, was sensitive to inhibition by corticosterone whereas that caused by small or moderate hemorrhage was not (41). Moreover, they found early immediate gene activation in magnocellular neurons of the supraoptic and paraventricular hypothalamic nuclei during hemorrhage and activation in the dorsomedial part of the hypothalamus by air-puff startle. Thus, this work differentiated feedback prone and feedback resistant mild stressors and observed signs of activation in magnocellular hypothalamic neurons in the case of the latter.

SIGNAL INTEGRATION BY CORTICOTROPE CELLS OF THE ANTERIOR PITUITARY GLAND AND THE VP-INDUCED ESCAPE FROM INHIBITION BY GLUCOCORTICOIDS

The stimulatory input to corticotrope cells is comprised of CRF and VP. A hypothalamic ACTH-inhibiting factor, atrial natriuretic peptide (ANP) has also been identified (39, 42). Finally, corticotrope cells are a well-established site of glucocorticoid feedback-inhibition (9, 10).

CRF

The widely accepted mechanism of action in the case of CRF is activation of CRF1 receptors and conservative coupling to the stimulatory G protein Gs. The consequent activation of transmembrane adenylyl cyclase(s) leads to increase of intracellular cAMP levels (43). The scenario of intracellular signaling downstream of cAMP is complex, as reviewed in detail elsewhere (44, 45). However, it is clear that intracellular Ca^{2+} signals are generated by CRF, and that the electrical activity of the cells ultimately determines the secretion of ACTH.

VP

In the case of VP, the consensus is the activation of V_{1b} receptors coupled to Gq and consequent activation of PLC, with ensuing increases of IP3 levels and PKC activity. Exactly which isoform(s) of PKC is (are) involved, is not entirely clear, but as phorbol esters can mimic most of the known actions of VP including the potentiation of the cAMP signal and the stimulation of ACTH secretion, the alpha and beta PKC-isoforms are the likely candidates. Once more, events after IP3 and PKC activation are too complex to be considered here (44, 46).

ANP

There is a signaling pathway to inhibit stimulated ACTH secretion through 3′5′guanosine monophosphate (cGMP), that is not shared with glucocorticoids (47). This pathway can be activated by ANP (48, 49), and may become functionally more prominent under conditions where the pituitary corticotropes are in "glucocorticoid escape" mode.

Glucocorticoids

Feedback inhibition in the HPA axis occurs in three time-domains; rapid <15 min, early delayed—within 20 min and up to 2 h, and delayed, beyond 2 h (9, 10, 50). With respect to the time-domains of feedback manifested in corticotropes: in contrast to a recent study (51), we have failed to observe a rapid inhibitory effect of glucocorticoids in dispersed rat anterior pituitary (52) or AtT-20D16:16 mouse corticotrope tumor cells (53). Thus, the focus of the studies outlined here was early-delayed feedback, which is the main inhibitory effect in the first 2 h after exposure to glucocorticoids. The properties of this inhibition have been reviewed extensively (9, 54, 55). In brief, it is mediated by Type II glucocorticoid receptors and is sensitive to inhibitors of transcription and translation, indicating a requirement of mRNA synthesis/processing and protein synthesis (52). What is the molecular and cellular basis of early- delayed inhibition? In AtT-20 cells, the balance of cAMP-dependent phosphorylation on the stress-axis-regulated-insert (STREX) type α-subunit of the large conductance Ca^{2+}-dependent K^{+}-channel (BK-channel) is shifted toward dephosphorylation. As a result, the channel is not inhibited by cAMP-dependent phosphorylation induced by CRF (56). Hence, the enhancement of intracellular free Ca^{2+} transients and the consequent stimulation of ACTH secretion by CRF are suppressed, reviewed in refs (54, 57). Subsequent work has implicated a protein phosphatase 2A-like enzyme as the mediator of glucocorticoid action (58), but definitive evidence is still outstanding. In contrast to AtT20 cells, blockade of BK-channels had no appreciable effect on the efficacy of corticosterone to inhibit CRF-induced ACTH secretion in cultured rat anterior pituitary cells (59). This is despite the fact, that BK channels play a prominent role in governing the firing pattern and the intracellular Ca^{2+} transients of anterior pituitary cells in culture (57) and that corticosterone has an inhibitory effect on the bursting activity of mouse corticotropes cells (44). Moreover, inhibition of agonist-induced ACTH release

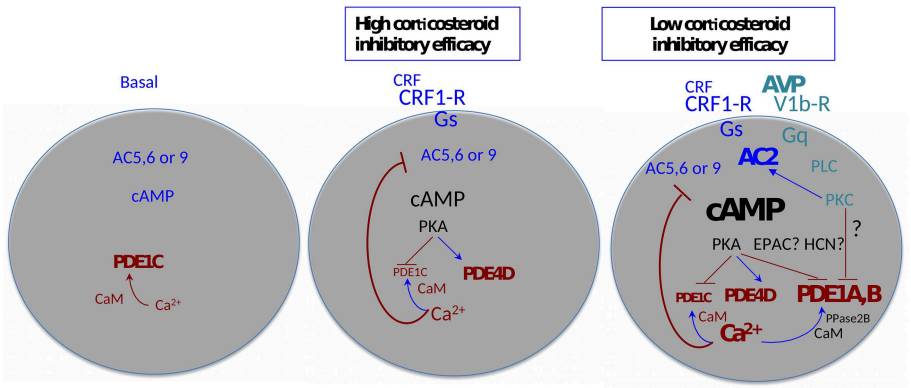

FIGURE 1 | Agonist-induced plasticity of cAMP signaling—multiple functional states of the adenohypophysial corticotroph cell. AC, adenylyl cyclase; CaM, calmodulin; EPAC, exchange factors directly activated by cAMP; HCN, Hyperpolarization activated cyclic nucleotide gated channels; PDE- cyclic nucleotide phosphodiesterase; PKA, cAMP dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; PPase2B, protein phosphatase 2B (*alias* calcineurin). Arrows indicate facilitation, T-bars indicate inhibition. The size and thickness of the lettering is proportional to the amplitude/activity of the denoted entity.

by ANP/cGMP, could be reversed by blockers of BK as well as other K^+ channels (60). In contrast, studies with a variety of K^+ inhibitors failed to identify conclusively the K^+ channel key for early delayed inhibition by corticosteroids (61). Others, using a similar panel of K^+ channel blockers, reported a role for *ether-á-go-go*-related gene K^+-channels as the mediators of glucocorticoid inhibition (62). However, these findings have not been confirmed by other, more direct methods, such as patch-clamp electrophysiology or gene-knock down. In summary, current evidence indicates that early-delayed corticosteroid feedback inhibition at the pituitary level is by glucocorticoid-induced protein(s) that facilitate the activation of K^+-channels. A similar conclusion was reached in a study of hippocampal pyramidal neurons (63). Significantly, this scenario implies that glucocorticoid inhibition is sensitive to depolarization of the membrane potential (59, 64).

One of the complicating factors in understanding signaling processes in corticotropes is the variety of the *in vitro* models used. It is clear that cultured cells, acutely dispersed cells, and pituitary slices may have different properties. Moreover, the temporal dynamics in perifused tissue are different to that seen in static systems. Finally, the concentrations of the effectors employed tend to vary from physiologic (CRF up to 0.3 nM, VP up to 3 nM) to the grossly aphysiologic.

With these considerations in mind, a detailed analysis of CRF-induced cAMP levels was carried out in the context of the inhibition of cAMP synthesis by intracellular free Ca^{2+}, and the efficacy of corticosteroid feedback inhibition of stimulated ACTH release. It transpired that CRF-induced cAMP synthesis is under feedback inhibition by Ca^{2+} derived from a ryanodine/caffeine sensitive intracellular pool (65). However, above 1 nM CRF, the effect of Ca^{2+} was no longer apparent. A similar "escape" of CRF-induced cAMP synthesis from Ca^{2+} inhibition could be induced by physiologic concentrations of VP, an effect also mimicked by phorbol-ester activation of protein kinase C (65). Analysis of cAMP hydrolysis indicated that while PDE4 isoforms were active when low concentrations of CRF were used, inhibitors of PDE1 were required for inhibiting cAMP breakdown when CRF was applied in combination with VP. When interpreted in the context of the diversity of cAMP signaling proteins (66, 67), the results indicate that physiologic concentrations of CRF activate a Ca^{2+} inhibited adenylyl cyclase such as AC5, 6, or 9, while in the presence of VP an adenylyl cyclase activated by protein kinase C such as AC2 or AC7 enters the fray (**Figure 1**). This latter cyclase is responsible for the markedly increased cAMP levels that require high-capacity, low-affinity phosphodiesterases to control cAMP signaling. By the combination of immunocytochemistry, Western blotting and RT-PCR, we concluded that AC7, PDE1a, and PDE1b were the best candidates underlying the switching of corticotropes to high cAMP levels in the context of stimulation by CRF and VP. Importantly, VP was present at concentrations that are only produced by magnocellular neurons under physiologic conditions.

It is pertinent to highlight here that the "amplitude modulation" of the cAMP signal outlined above has potentially important consequences: whilst PKA is maximally active at 1 μM cAMP, other effectors such as EPACS, hyperpolarization-activated cyclic nucleotide–gated (HCN) channels, and cyclic nucleotide gated channels all require >10 μM cAMP for activity. Thus, a whole plethora of cAMP signaling systems is potentially mobilized in corticotrope cells in the presence of high (magnocellular) concentrations of VP that are not active at levels of stimulation that can be achieved *via* parvicellular CRF/VP. It is also important to reiterate here that the effects of VP on cAMP synthesis are conditional to the presence of CRF.

Several of the inferences in the aforementioned studies were made on the basis of the application of pharmacologic agents or the use of antibodies. It later transpired, that the antibodies used to localize AC2 in the anterior pituitary gland were unreliable, similarly, an antibody against AC5/6 gave also inconsistent results (68). More recently, mRNA-seq analyses of single anterior pituitary cells have been published (69). The data show selective enrichment of AC2 in corticotrope cells, in contrast, the levels of AC7 mRNA in anterior pituitary cells are

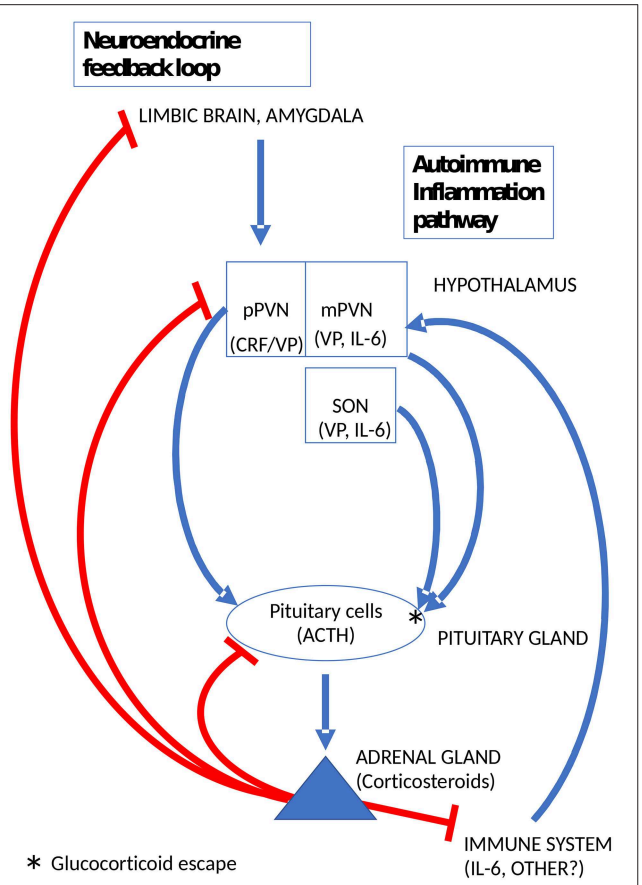

FIGURE 2 | Glucocorticoid escape at the pituitary gland induced by magnocellular VP to mobilize the endogenous immunosuppressant activity of adrenal corticosteroids. pPVN, parvicellular medial dorsal part of the hypothalamic paraventricular nucleus; mPVN lateral magnocellular part of the PVN; SON, supraoptic hypothalamic nucleus.

very low. All three Ca^{2+}-inhibited ACs, AC5, 6, and 9 appear to be expressed in corticotropes. Moreover, the highest relative levels of PDE1a and PDE1b appear to be associated with cells identified as corticotropes.

Taken together, the molecular machinery of Ca^{2+} feedback on cAMP biosynthesis and for achieving the VP-mediated boost of cellular cAMP to levels that address EPACS, HCNs and CNGs is present in corticotropes. Finally, is attractive to speculate here, that high VP overrides early feedback through depolarization of the membrane potential by activation of an HCN channel (70, 71).

The analysis of the efficacy of corticosterone to inhibit CRF-induced ACTH release showed interesting parallels with the characteristics of the inhibition of cAMP synthesis by Ca^{2+}(61). In the physiologic range for CRF, corticosterone inhibition was highly efficient, while at supraphysiological CRF the inhibitory efficacy was markedly reduced. Application of the PDE4 blocker rolipram or high, physiologic levels of VP with physiologic concentrations of CRF also markedly reduced the inhibitory efficacy of corticosterone. In essence, irrespective of how intracellular cAMP levels were elevated above 20 μM, glucocorticoid efficacy was progressively reduced. Thus, if the agonists by-pass the Ca^{2+} inhibitory feedback on adenylyl cyclase, the efficacy of glucocorticoid inhibition is dramatically reduced. These observations link the VP mediated switch of low cAMP signaling induced by CRF to high cAMP, with the escape from glucocorticoid feedback at the pituitary level. A summary cartoon of the corticosteroid sensitive and insensitive states of anterior pituitary corticotrope cells is shown in **Figure 1**.

WHEN IS GLUCOCORTICOID ESCAPE OF BIOLOGICAL IMPORTANCE?

The pronounced deleterious effects of long-term corticosteroid excess on body homeostasis indicate that glucocorticoid escape is encountered in life-threatening situations and is likely to be of relatively short duration. The classic clinical examples of persistent glucocorticoid non-suppression are Cushing's disease (72) and melancholic depression (12).

Autoimmune Inflammation

In preclinical models of autoimmune inflammation, such as experimental autoimmune encephalomyelitis and adjuvant-induced arthritis, a sustained rise of plasma corticosteroids lasting several days is crucial for survival (73). Indeed, it is widely recognized that adrenal corticosteroids are important facilitators of endogenous immunosuppression (74). The only way this can occur is by escape from glucocorticoid feedback inhibition. Several lines of evidence indicate, that the sustained and vital increase of corticosteroids in preclinical autoimmune models is mediated by VP (75, 76). Moreover, the data of Suzuki etal. (76), indicated at the level of VP expression rather than mRNA, that magnocellular vasopressinergic neurons become activated during autoimmune inflammation. It is relevant to recall here that VP synthesis is not closely controlled by adrenal corticosteroids in magnocellular neurons (35) and that the effects of low levels of CRF on corticotropes are amplified dramatically by VP (65, 77). Thus, activation of magnocellular VP neurons can bypass glucocorticoid feedback in the CNS and induce escape from glucocorticoid feedback at the pituitary level to support the sustained secretion of corticosteroids. The trigger for the activation of hypothalamic neurons during autoimmune inflammation is unknown. It is plausibly a cytokine, above all, interleukin-6 (IL-6) (78) known to activate the HPA axis in humans as well as rodents (79). However, the exact mechanism by which this occurs remains to be clarified (80). Intriguingly, magnocellular VP neurons also produce IL-6 (81). Moreover, increases of IL-6 levels in magnocellular neurons were observed after various types of stress paradigms (82). Whether or not this IL-6 is involved in the regulation VP release e.g., as a paracrine mediator, is unknown. A summary diagram of the by-pass of glucocorticoid feedback in the HPA axis through the activation of magnocellular VP neurons by immune mediators is shown in **Figure 2**.

High Intensity Exercise and Hyponatremia

Work by Deuster et al. (83) reported that high-intensity exercise results in dexamethasone non-suppression in men as well as women. One of the threats of high-intensity exercise is hyponatremia (84), which may be lethal due to the development of hypotonic encephalopathy (85). Increases of plasma VP are prominent under these conditions and it has been argued that exercise-associated hypernatremia is a case of inappropriate VP secretion (SIADH). It is likely, that IL-6 released from muscle cells during exercise is a trigger for VP secretion. Additionally, hyperhydration by the subjects during exercise may contribute to hyponatremia. In the pituitary portal system, where VP levels are considerably higher than in the periphery, the levels of vasopressin are sufficient to induce glucocorticoid escape at the pituitary level. The ensuing higher levels of ACTH would enhance aldosterone secretion by zona glomerulosa cells and improve sodium retention. In addition, VP has a direct action to stimulate aldosterone secretion in rat (86) as well as human zona glomerulosa cells (87). Thus, while exercise may symptomatically mimic SIADH in that plasma levels of VP increase despite hypoosmolarity, it may be a fluid-volume preservation reflex that simultaneously promotes sodium retention by bringing about glucocorticoid non-suppression at the pituitary level.

SUMMARY

In a day-to-day setting, the activity of the HPA axis is under powerful feedback inhibitory control. The situation during certain types of stress may be different. In a seminal review, Munck et al. (88) proposed that the physiologic rationale of stress-induced glucocorticoid production was to dampen and reset the production of powerful immune mediators induced by the stress response. Three decades, two dozen cytokines and a few microbiomes later, this concept is stronger than ever. In particular, the emergence of IL-6 as a pleiotropic stress-responsive protein and its activation of the hypothalamic CRF and VP systems is of particular note. If corticosteroids are controlled by strict neuroendocrine feedback tailored for stress-free and/or casual mild stress conditions, they could not be deployed to combat stress with a substantial inflammatory component. Increasingly, it appears that most types of stress also mobilize inflammatory mediators (89, 90). Similarly, given the threat of hyponatremia in severe hypovolemia, overriding inhibitory feedback to allow sodium retention through aldosterone may be key to survival. Therefore, feedback inhibition has to be plastic. Indeed, the functional neuroanatomy, as well as the molecular and cellular elements underpinning the plasticity of corticosteroid feedback inhibition are clearly apparent in the hypothalamo-hypophysial system. Where sustained secretion of adrenal corticosteroids is vital for survival, the evidence points to a critical role of magnocellular VP to induce glucocorticoid escape at the pituitary level. Whether or not this insight can be exploited therapeutically remains to be explored.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

I would like to thank Dr. Paul LeTissier and Dr. Nicola Romano for helpful discussions regarding pituitary gene-expression.

REFERENCES

1. Réthelyi M, Szentágothai. *Functional Anatomy: Anatomy, Histology and Embryology for Medical and Dental Students*. Budapest: Medicina (2018).
2. Budnik B, Levy E, Harmange G, Slavov N. SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biol.* (2018) 19:161. doi: 10.1186/s13059-018-1547-5
3. Fink G. 60 years of neuroendocrinology: memoir: harris' neuroendocrine revolution: of portal vessels and self-priming. *J Endocrinol.* (2015) 226:T13–24. doi: 10.1530/JOE-15-0130
4. Kiss JZ, Palkovits M, Záborszky L, Tribollet E, Szabó D, Makara GB. Quantitative histological studies on the hypothalamic paraventricular nucleus in rats. II. number of local and certain afferent nerve terminals. *Brain Res.* (1983) 265:11–20. doi: 10.1016/0006-8993(83)91328-8
5. Sawchenko PE, Swanson LW. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. *J Comp Neurol.* (1983) 218:121–44. doi: 10.1002/cne.902180202
6. Ziegler DR, Herman JP. Neurocircuitry of stress integration: anatomical pathways regulating the hypothalamo-pituitary-adrenocortical axis of the rat. *Integr Comp Biol.* (2002) 42:541–51. doi: 10.1093/icb/42.3.541
7. Mcklveen JM, Myers B, Herman JP. The medial prefrontal cortex: coordinator of autonomic, neuroendocrine and behavioural responses to stress. *J Neuroendocrinol.* (2015) 27:446–56. doi: 10.1111/jne.12272
8. Myers B, Scheimann JR, Franco-Villanueva A, Herman JP. Ascending mechanisms of stress integration: Implications for brainstem regulation of neuroendocrine and behavioral stress responses. *Neurosci Biobehav Rev.* (2017) 74:366–75. doi: 10.1016/j.neubiorev.2016.05.011
9. Keller-Wood MB, Dallman MF. Corticosteroid inhibition of ACTH secretion. *Endocr Rev.* (1984) 5:1–23. doi: 10.1210/edrv-5-1-1
10. De Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci.* (2005) 6:463–75. doi: 10.1038/nrn1683
11. Ising M, Horstmann S, Kloiber S, Lucae S, Binder EB, Kern N, etal. Combined dexamethasone/corticotropin releasing hormone test predicts treatment response in major depression–a potential biomarker? *Biol Psychiatry.* (2007) 62:47–54. doi: 10.1016/j.biopsych.2006.07.039
12. Heuser I, Yassouridis A, Holsboer F. The combined dexamethasone/CRH test: a refined laboratory test for psychiatric disorders. *J Psychiatr Res.* (1994) 28:341–56. doi: 10.1016/0022-3956(94)90017-5
13. Cole MA, Kim PJ, Kalman BA, Spencer RL. Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. *Psychoneuroendocrinology.* (2000) 25:151–67. doi: 10.1016/S0306-4530(99)00045-1
14. Bichet DG. The posterior pituitary. In: Melmed S, editor. *The Pituitary*. San Diego, CA: Academic Press (2017). p. 251–88.
15. Brownstein MJ, Russell JT, Gainer H. Synthesis, transport, and release of posterior pituitary hormones. *Science.* (1980) 207:373–8. doi: 10.1126/science.6153132
16. Ludwig M, Leng G. Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci.* (2006) 7:126–36. doi: 10.1038/nrn1845

17. Morgenthaler NG, Struck J, Jochberger S, Dunser MW. Copeptin: clinical use of a new biomarker. *Trends Endocrinol Metab.* (2008) 19:43–9. doi: 10.1016/j.tem.2007.11.001
18. Christ-Crain M, Fenske W. Copeptin in the diagnosis of vasopressin-dependent disorders of fluid homeostasis. *Nat Rev Endocrinol.* (2016) 12:168–76. doi: 10.1038/nrendo.2015.224
19. Antoni FA. Novel ligand specificity of pituitary vasopressin receptors in the rat. *Neuroendocrinology.* (1984) 39:186–8. doi: 10.1159/000123976
20. Roper JA, O'carroll AM, Young WS III, Lolait SJ. The vasopressin AVP1b receptor: Molecular and pharmacological studies. *Stress.* (2011) 14:98–115. doi: 10.3109/10253890.2010.512376
21. Scharrer B. Neurosecretion: beginnings and new directions in neuropeptide research. *Annu Rev Neurosci.* (1987) 10:1–17. doi: 10.1146/annurev.neuro.10.1.1
22. Holmes MC, Antoni FA, Aguilera G, Catt KJ. Magnocellular axons in passage through the median eminence release vasopressin. *Nature.* (1986) 319:326–9. doi: 10.1038/319326a0
23. Morris JF, Pow DV. Capturing and quantifying the exocytotic event. *J Exp Biol.* (1988) 139:81–103.
24. Engelmann M, Landgraf R, Wotjak CT. The hypothalamic-neurohypophysial system regulates the hypothalamic-pituitary-adrenal axis under stress: an old concept revisited. *Front Neuroendocrinol.* (2004) 25:132–49. doi: 10.1016/j.yfrne.2004.09.001
25. Antoni FA, Fink G, Sheward WJ. Corticotrophin-releasing peptides in rat hypophysial portal blood after paraventricular lesions: a marked reduction in the concentration of 41-residue corticotrophin releasing factor, but no change in vasopressin. *J Endocrinol.* (1990) 125:175–83. doi: 10.1677/joe.0.1250175
26. Léránth C, Antoni FA, Palkovits M. Ultrastructural demonstration of ovine CRF-like immunoreactivity (oCRF-LI) in the rat hypothalamus: processes of magnocellular neurons establish membrane specializations with parvocellular neurons containing oCRF-LI. *Regulatory Peptides.* (1983) 6:179–88. doi: 10.1016/0167-0115(83)90011-3
27. Liposits Z, Paull WK, Sétáló G, Vigh S. Evidence for local corticotropin releasing factor (CRF)-immunoreactive neuronal circuits in the paraventricular nucleus of the rat hypothalamus. *Histochemistry.* (1985) 83:5–16. doi: 10.1007/BF00495294
28. Plotsky PM, Bruhn TO, Vale W. Central modulation of immunoreactive corticotropin-releasing factor secretion by arginine vasopressin. *Endocrinology.* (1985) 115:1639–41. doi: 10.1210/endo-115-4-1639
29. Whitnall MH, Mezey E, Gainer H. Co-localization of corticotropin-releasing factor and vasopressin in median eminence neurosecretory vesicles. *Nature.* (1985) 317:248–50. doi: 10.1038/317248a0
30. Kiss JZ, Bertini LT. The CRF neurosecretory vesicle: vasopressin-dependent changes in vesicle size after adrenalectomy. *Brain Res.* (1992) 597:353–7. doi: 10.1016/0006-8993(92)91495-Z
31. Makara GB, Antoni FA, Stark E, Kárteszi M. Hypothalamic organization of CRF containing structures. *Neuroendocrine Perspect.* (1984) 3:71–110. doi: 10.1016/B978-0-444-90377-8.50006-5
32. Antoni FA. Hypothalamic regulation of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocr Rev.* (1986) 7:351–78. doi: 10.1210/edrv-7-4-351
33. Knepel W, Przewlocki R, Nutto D, Herz A. Foot shock stress-induced release of vasopressin in adenohypophysectomized and hypophysectomized rats. *Endocrinology.* (1985) 117:292–9. doi: 10.1210/endo-117-1-292
34. Kovács KJ, Földes A, Sawchenko PE. Glucocorticoid negative feedback selectively targets vasopressin transcription in parvocellular neurosecretory neurons. *J Neurosci.* (2000) 20:3843–52. doi: 10.1523/JNEUROSCI.20-10-03843.2000
35. Raff H. Glucocorticoid inhibition of neurohypophysial vasopressin secretion. *Am J Physiol.* (1987) 252:R635–44. doi: 10.1152/ajpregu.1987.252.4.R635
36. Kiss JZ, Van Eekelen JA, Reul JM, Westphal HM, De Kloet ER. Glucocorticoid receptor in magnocellular neurosecretory cells. *Endocrinology.* (1988) 122:444–9. doi: 10.1210/endo-122-2-444
37. Holmes MC, Antoni FA, Catt KJ, Aguilera G. Predominant release of vasopressin vs. corticotropin releasing factor from the isolated median eminence after adrenalectomy. *Neuroendocrinology.* (1986) 43:245–51. doi: 10.1159/000124533
38. Makara GB, Mergl Z, Zelena D. The role of vasopressin in hypothalamo-pituitary-adrenal axis activation during stress: an assessment of the evidence. *Ann N Y Acad Sci.* (2004) 1018:151–61. doi: 10.1196/annals.1296.018
39. Antoni FA. Vasopressinergic control of anterior pituitary adrenocorticotropin secretion comes of age. *Front Neuroendocrinol.* (1993) 14:76–122. doi: 10.1006/frne.1993.1004
40. Thrivikraman KV, Plotsky PM. Absence of glucocorticoid negative feedback to moderate hemorrhage in conscious rats. *Am J Physiol.* (1993) 264:E497–503. doi: 10.1152/ajpendo.1993.264.4.E497
41. Thrivikraman KV, Nemeroff CB, Plotsky PM. Sensitivity to glucocorticoid-mediated fast-feedback regulation of the hypothalamic-pituitary-adrenal axis is dependent upon stressor specific neurocircuitry. *Brain Res.* (2000) 870:87–101. doi: 10.1016/S0006-8993(00)02405-7
42. Engler D, Redei E, Kola I. The corticotropin-release inhibitory factor hypothesis: a review of the evidence for the existence of inhibitory as well as stimulatory hypophysiotropic regulation of adrenocorticotropin secretion and biosynthesis. *Endocr Rev.* (1999) 20:460–500. doi: 10.1210/edrv.20.4.0376
43. Dautzenberg FM, Grigoriadis DE, Hauger RL, Steckler T, Vale WW. Corticotropin-releasing factor receptors: CRF1 receptor. *IUPHAR/BPS Guide to Pharmacology*. Available online at: http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=212andfamilyId=219andfamilyType=GPCR~(2019).
44. Duncan PJ, Tabak J, Ruth P, Bertram R, Shipston MJ. Glucocorticoids inhibit CRH/AVP-evoked bursting activity of male murine anterior pituitary corticotrophs. *Endocrinology.* (2016) 157:3108–21. doi: 10.1210/en.2016-1115
45. Fletcher PA, Zemkova H, Stojilkovic SS, Sherman A. Modeling the diversity of spontaneous and agonist-induced electrical activity in anterior pituitary corticotrophs. *J Neurophysiol.* (2017) 117:2298–311. doi: 10.1152/jn.00948.2016
46. Lee AK, Tse FW, Tse A. Arginine vasopressin potentiates the stimulatory action of CRH on pituitary corticotropes via a protein kinase C-dependent reduction of the background TREK-1 current. *Endocrinology.* (2015) 156:3661–72. doi: 10.1210/en.2015-1293
47. Antoni FA, Dayanithi G. Evidence for distinct glucocorticoid and guanine 3′,5′ monophosphate effected inhibition of secretagogue stimulated ACTH release *in vitro*. *Endocrinology.* (1990) 126:1355–60. doi: 10.1210/endo-126-3-1355
48. Dayanithi G, Antoni FA. Atriopeptins are potent inhibitors of ACTH secretion by rat anterior pituitary cells *in vitro*: involvement of the atriopeptin receptor domain of membrane bound guanylyl cyclase. *J Endocrinol.* (1990) 125:39–44. doi: 10.1677/joe.0.1250039
49. Antoni FA, Hunter EFM, Lowry PJ, Noble JM, Seckl JR. Atriopeptin: an endogenous corticotropin-release inhibiting hormone. *Endocrinology.* (1992) 130:1753–5. doi: 10.1210/endo.130.3.1311248
50. Osterlund CD, Rodriguez-Santiago M, Woodruff ER, Newsom RJ, Chadayammuri AP, Spencer RL. Glucocorticoid fast feedback inhibition of stress-induced ACTH secretion in the male rat: rate independence and stress-state resistance. *Endocrinology.* (2016) 157:2785–98. doi: 10.1210/en.2016-1123
51. Deng Q, Riquelme D, Trinh L, Low MJ, Tomic M, Stojilkovic S, etal. Rapid glucocorticoid feedback inhibition of acth secretion involves ligand-dependent membrane association of glucocorticoid receptors. *Endocrinology.* (2015) 156:3215–27. doi: 10.1210/EN.2015-1265
52. Dayanithi G, Antoni FA. Rapid as well as delayed inhibitory effects of glucocorticoid hormones on pituitary adrenocorticotropic hormone release are mediated by type II glucocorticoid receptors and require newly synthesized messenger ribonucleic acid as well as protein. *Endocrinology.* (1989) 125:308–13. doi: 10.1210/endo-125-1-308
53. Woods MD, Shipston MJ, Mullens EL, Antoni FA. Pituitary corticotrope tumor (AtT20) cells as a model system for the study of early inhibition by glucocorticoids. *Endocrinology.* (1992) 131:2873–80. doi: 10.1210/endo.131.6.1332850
54. Antoni FA. Calcium checks cyclic AMP — mechanism of corticosteroid feedback in adenohypophysial corticotrophs. *J Neuroendocrinol.* (1996) 8:659–72. doi: 10.1046/j.1365-2826.1996.05152.x
55. Osterlund C, Spencer RL. Corticosterone pretreatment suppresses stress-induced hypothalamic-pituitary-adrenal axis activity via multiple actions that

vary with time, site of action, and de novo protein synthesis. *J Endocrinol.* (2011) 208:311–22. doi: 10.1530/JOE-10-0413
56. Shipston MJ, Kelly JS, Antoni FA. Glucocorticoids block protein kinase A inhibition of calcium-activated potassium channels. *J Biol Chem.* (1996) 271:9197–200. doi: 10.1074/jbc.271.16.9197
57. Shipston MJ. Control of anterior pituitary cell excitability by calcium-activated potassium channels. *Mol Cell Endocrinol.* (2018) 463:37–48. doi: 10.1016/j.mce.2017.06.003
58. Tian L, Knaus HG, Shipston MJ. Glucocorticoid regulation of calcium-activated potassium channels mediated by serine/threonine protein phosphatase. *J Biol Chem.* (1998) 273:13531–6. doi: 10.1074/jbc.273.22.13531
59. Lim MC, Shipston MJ, Antoni FA. Depolarisation counteracts glucocorticoid inhibition of adenohypophysial corticotroph cells. *Br J Pharmacol.* (1998) 124:1735–43. doi: 10.1038/sj.bjp.0702024
60. Antoni FA, Dayanithi G. Blockage of K^+ channels reverses the inhibition of secretagogue stimulated ACTH release by atriopeptin. *J Endocrinol.* (1990) 126:183–91. doi: 10.1677/joe.0.1260183
61. Lim MC, Shipston MJ, Antoni FA. Posttranslation al modulation of glucocorticoid inhibition at the pituitary level. *Endocrinology.* (2002) 143:3796–801. doi: 10.1210/en.2002-220489
62. Yamashita M, Oki Y, Iino K, Hayashi C, Matsushita F, Faje A, etal. The role of ether-à-go-go-related gene K(+) channels in glucocorticoid inhibition of adrenocorticotropin release by rat pituitary cells. *Regul Pept.* (2009) 152:73–8. doi: 10.1016/j.regpep.2008.09.002
63. Joëls M, De Kloet ER. Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. *Science.* (1989) 245:1502–5. doi: 10.1126/science.2781292
64. Woods MD, Shipston MJ, Mcferran B, Guild SB, Antoni FA. Early glucocorticoid inhibition of hormone release in pituitary corticotrope cells is voltage dependent. *Ann N Y Acad Sci.* (1994) 746:456–9. doi: 10.1111/j.1749-6632.1994.tb39284.x
65. Antoni FA, Sosunov AA, Haunso A, Paterson JM, Simpson J. Short-term plasticity of cyclic adenosine 3′,5′-monophosphate signaling in anterior pituitary corticotrope cells: the role of adenylyl cyclase isotypes. *Mol Endocrinol.* (2003) 17:692–703. doi: 10.1210/me.2002-0369
66. Antoni FA. Molecular diversity of cyclic AMP signaling. *Front Neuroendocrinol.* (2000) 21:103–32. doi: 10.1006/frne.1999.0193
67. Dessauer CW, Watts VJ, Ostrom RS, Conti M, Dove S, Seifert R. International union of basic and clinical pharmacology. ci structures and small molecule modulators of mammalian adenylyl cyclases *Pharmacol Rev.* (2017) 69:93–139. doi: 10.1124/pr.116.013078
68. Antoni FA, Wiegand UK, Black J, Simpson J. Cellular localisation of adenylyl cyclase: a post-genome perspective. *Neurochem Res.* (2006) 32:287–95. doi: 10.1007/s11064-005-9019-1
69. Cheung LYM, George AS, McGee SR, Daly AZ, Brinkmeier ML, Ellsworth BS, etal. Single-cell RNA sequencing reveals novel markers of male pituitary stem cells and hormone-producing cell types. *Endocrinology.* (2018) 159:3910–24. doi: 10.1210/en.2018-00750
70. Herrmann S, Stieber J, Stockl G, Hofmann F, Ludwig A. HCN4 provides a 'depolarization reserve' and is not required for heart rate acceleration in mice. *EMBO J.* (2007) 26:4423–32. doi: 10.1038/sj.emboj.7601868
71. Raggenbass M. Overview of cellular electrophysiological actions of vasopressin. *Eur J Pharmacol.* (2008) 583:243–54. doi: 10.1016/j.ejphar.2007.11.074
72. Lonser RR, Nieman L, Oldfield EH. Cushing's disease: pathobiology, diagnosis, and management. *J Neurosurg.* (2017) 126:404–17. doi: 10.3171/2016.1.JNS152119
73. Mason D. Genetic variation in the stress response susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. *Immunol Today.* (1991) 12:57–60. doi: 10.1016/0167-5699(91)90158-P
74. Morand EF, Leech M. Hypothalamic-pituitary-adrenal axis regulation of inflammation in rheumatoid arthritis. *Immunol Cell Biol.* (2001) 79:395–9. doi: 10.1046/j.1440-1711.2001.01028.x
75. Chowdrey HS, Larsen PJ, Harbuz MS, Jessop DS, Aguilera G, Eckland DJ, etal. Evidence for arginine vasopressin as the primary activator of the HPA axis during adjuvant-induced arthritis. *Br J Pharmacol.* (1995) 116:2417–24. doi: 10.1111/j.1476-5381.1995.tb15089.x
76. Suzuki H, Onaka T, Kasai M, Kawasaki M, Ohnishi H, Otsubo H, etal. Response of arginine vasopressin-enhanced green fluorescent protein fusion gene in the hypothalamus of adjuvant-induced arthritic rats. *J Neuroendocrinol.* (2009) 21:183–90. doi: 10.1111/j.1365-2826.2009.01841.x
77. Gillies GE, Linton EA, Lowry PJ. Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature.* (1982) 299:355–7. doi: 10.1038/299355a0
78. Kishimoto T, Kang S, Tanaka T. *IL-6: A New Era for the Treatment of Autoimmune Inflammatory Diseases.* Tokyo: Springer (2015). doi: 10.1007/978-4-431-55651-0_11
79. Mastorakos G, Chrousos GP, Weber JS. Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans. *J Clin Endocrinol Metab.* (1993) 77:1690–4. doi: 10.1210/jc.77.6.1690
80. Swart RM, Hoorn EJ, Betjes MG, Zietse R. Hyponatremia and inflammation: the emerging role of interleukin-6 in osmoregulation. *Nephron Physiol.* (2011) 118:45–51. doi: 10.1159/000322238
81. Ghorbel MT, Sharman G, Leroux M, Barrett T, Donovan DM, Becker KG, etal. Microarray Analysis Reveals Interleukin-6 as a novel secretory product of the hypothalamo-neurohypophyseal system. *J Biol Chem.* (2003) 278:19280–5. doi: 10.1074/jbc.M209902200
82. Jankord R, Zhang R, Flak JN, Solomon MB, Albertz J, Herman JP. Stress activation of IL-6 neurons in the hypothalamus. *Am J Physiol Regul Integr Comp Physiol.* (2010) 299:R343–351. doi: 10.1152/ajpregu.00131.2010
83. Deuster PA, Petrides JS, Singh A, Lucci EB, Chrousos GP, Gold PW. High intensity exercise promotes escape of adrenocorticotropin and cortisol from suppression by dexamethasone: sexually dimorphic responses. *J Clin Endocrinol Metab.* (1998) 83:3332–8. doi: 10.1210/jc.83.9.3332
84. Rosner MH, Kirven J. Exercise-associated hyponatremia. *Clin J Am Soc Nephrol.* (2007) 2:151–61. doi: 10.2215/CJN.02730806
85. Siegel AJ, Verbalis JG, Clement S, Mendelson JH, Mello NK, Adner M, etal. Hyponatremia in marathon runners due to inappropriate arginine vasopressin secretion. *Am J Med.* (2007) 120: e411–67. doi: 10.1016/j.amjmed.2006.10.027
86. Balla T, Enyedi P, Spät A, Antoni FA. Pressor-type vasopressin receptors in the adrenal cortex: properties of binding, effects on phosphoinositide metabolism and aldosterone secretion. *Endocrinology.* (1985) 117:421–3. doi: 10.1210/endo-117-1-421
87. Guillon G, Trueba M, Joubert D, Grazzini E, Chouinard L, Cote M, etal. Vasopressin stimulates steroid secretion in human adrenal glands: comparison with angiotensin-II effect. *Endocrinology.* (1995) 136:1285–95. doi: 10.1210/endo.136.3.7867583
88. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev.* (1984) 5:25–44. doi: 10.1210/edrv-5-1-25
89. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev.* (2000) 21:55–89. doi: 10.1210/er.21.1.55
90. Pariante CM. The year of immunopsychiatry: a special issue that foresaw the future. *Psychoneuroendocrinology.* (2019) 103:49–51. doi: 10.1016/j.psyneuen.2019.01.002

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 26 July 2019
doi: 10.3389/fendo.2019.00491

Stability and Turnover of the ACTH Receptor Complex

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Glucocorticoid production in mammals is principally regulated by the action of the pituitary hormone adrenocorticotropin (ACTH) acting on its cognate membrane receptor on the zona fasciculata cells of the adrenal cortex. The receptor for ACTH consists of two essential components, a small seven transmembrane domain G protein-coupled receptor of the melanocortin receptor subgroup known as the melanocortin 2 receptor (MC2R) and a small single transmembrane domain protein that adopts a antiparallel homodimeric form and which is known as the melanocortin 2 receptor accessory protein (MRAP). MRAP is essential for the trafficking of the MC2R to the cell surface as well as being required for receptor responsiveness to ACTH at physiological concentrations—probably by facilitating ACTH binding, but possibly also by supporting G protein interaction with the MC2R. A number of studies have shown that ACTH stimulates the expression of functional receptor at the cell surface and the transcription of both MC2R and MRAP mRNA. However, the time course of these transcriptional effects differs such that MRAP is expressed relatively rapidly whereas MC2R transcription responds much more slowly. Furthermore, recent data suggests that MRAP protein is turned over with a short half-life whereas MC2R has a significantly longer half-life. These findings imply that these two ACTH receptor proteins have distinct trajectories and that it is likely that MRAP-independent MC2R is present at the cell surface. In such a situation newly transcribed and translated MRAP could enable the rapid recruitment of functional receptor at the plasma membrane without the need for new MC2R translation. This may be advantageous in circumstances of significant stress in that the potentially complex and perhaps inefficient process of *de novo* MC2R translation, folding, post-translational modification and trafficking can be avoided.

Keywords: ACTH receptor, adrenal cortex, glucocorticoid, G protein-coupled receptor, adrenocorticotropin, melanocortin receptor

OPEN ACCESS

Edited by:
László Hunyady, Semmelweis University, Hungary

Reviewed by:
Rostislav Turecek, Academy of Sciences of the Czech Republic (ASCR), Czechia
Patricia M. Hinkle, Medical Center, University of Rochester, United States

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *13 February 2019*
Accepted: *08 July 2019*
Published: *26 July 2019*

Citation:
Clark AJL and Chan L (2019) Stability and Turnover of the ACTH Receptor Complex. Front. Endocrinol. 10:491. doi: 10.3389/fendo.2019.00491

The pituitary-adrenal axis provides a vital mechanism for protection against stress, allowing higher centers to rapidly generate increases in circulating glucocorticoids. These in turn adjust the metabolic and immunological functions of the body to cope with the altered environment. Failure of this system is potentially fatal. It is surprising therefore that there is not greater redundancy among the components of this system. For example, the key messenger hormone, adrenocorticotropin (ACTH) is the product of a single gene and is secreted from a single population of corticotroph cells in a single anatomical organ—the anterior pituitary gland. ACTH in turn acts

through a single receptor located almost entirely on the surface of cells of the two adrenal glands, resulting in the production of a single glucocorticoid hormone—cortisol or corticosterone. Although the ACTH receptor was one of the first receptors to be shown to signal through stimulation of cyclic AMP (1), and one of the first receptors for which ligand binding was demonstrated (2), the molecular characterization of this entity was relatively delayed. Protein purification approaches were largely unsuccessful, and it was not until a gene was identified through reduced stringency PCR which was shown to be closely related to a gene encoding a receptor for α-melanocyte stimulating hormone (α-MSH). This gene was expressed almost exclusively in the adrenal cortex and hence was described as the melanocortin 2 receptor (MC2R) and proposed to encode the ACTH receptor (3). Subsequent demonstration of mutations in this gene in patients with ACTH resistance lent support to this identification (4, 5).

With most receptors for which a gene or cDNA is identified in this way the usual practice is to transfect the candidate DNA into a cell type that lacks the receptor in question and to show the cell acquires the characteristics of the receptor. In the case of the ACTH receptor however this proved very difficult. Some evidence of expression could be found in cell types that already possessed MSH receptors (3, 6), but expression in a true null cell devoid of any MSH receptor was not achievable.

The first evidence that the MC2R required a co-factor came from the demonstration that it could be expressed in adrenal cell lines that lacked endogenous ACTH receptors—implying that there was something else about the adrenal cell required for successful expression (7). However, it was not until a new gene was found that was mutated in patients with ACTH resistance but a normal MC2R gene that a solution to this problem was identified. This new gene encoded a small single transmembrane domain protein that when co-expressed with the MC2R in transfected cells conferred typical ACTH responsiveness to those cells. This gene was named the melanocortin 2 receptor accessory protein—MRAP (8).

Evidence from a number of labs showed that the MRAP protein interacted with the MC2R as a homodimer and then trafficked with the receptor to the plasma membrane (9, 10). This appeared to be the primary function of MRAP in that without MRAP, the MC2R protein accumulated at the ER and did not appear at the cell surface.

At the cell surface MRAP maintained an association with the receptor protein and the evidence suggests it is required for ACTH binding or possibly in signal transduction (11, 12). Perhaps the most unusual phenomenon associated with MRAP is that it exists as an antiparallel homodimer spanning the plasma membrane such that one N-terminus is extracellular and one is intracellular (9). This topology is probably unique in eukaryotic biology, and suggests a very unusual mode of action that remains poorly understood.

These aspects of MRAP have been reviewed elsewhere (13, 14) and are not the prime focus of this paper. Instead, we will consider the dynamic aspects of MRAP which may challenge the notion that it is the "loyal and lifelong" partner of the MC2R as may be assumed, or whether it has a more complex and perhaps transient role with its partner protein.

INDUCTION OF MC2R mRNA BY ACTH

It was recognized some years before the ACTH receptor was cloned and characterized that ACTH could induce increased responsiveness to itself and an increased number of receptors in the adrenal. Thus, for example, Durand and Locatelli (15) demonstrated in rabbits that ACTH treatment increased the number of its adrenal receptors. Similar findings were reported from fetal sheep adrenals (16). Penhoat et al. (17) showed that 48 h treatment with physiological concentrations of ACTH increased ACTH ligand binding on bovine adrenal cells four-fold.

Following the cloning of the *MC2R* several groups explored the influence of ACTH and other stimuli on *MC2R* mRNA. Thus Mountjoy et al. (18) showed a 6-fold increase in *Mc2r* mRNA in mouse Y1 cells and a 2-fold increase in human H-295 cells after 24 h of treatment with ACTH. Lebrethon et al. (19) found a 21-fold increase in *MC2R* mRNA and a 4-fold increase in ACTH binding in human primary adrenal cells, initial responses being seen within 12 h of treatment, and similar changes after 48 h treatment with ACTH in human fetal adrenal cells (20). Rehman et al. (21) showed induction of *MC2R* mRNA after ACTH after 6 h in cultured human fetal adrenal cells. More recently Liu et al described the effect of restraint stress or ACTH injection on rat adrenal *Mc2r* mRNA over a short time period and failed to show any changes over the initial 4 h (in response to restraint) or over the first 60 min in response to ACTH (22). These and other similar studies are summarized in **Table 1**.

INDUCTION OF MRAP mRNA BY ACTH

As the *MRAP* gene was only identified in 2005 these earlier studies did not examine its response to ACTH and related stimuli, and it is only in later studies that both *MC2R* and *MRAP* gene expression in the same model has been reported.

Thus in their study Liu et al. (22) demonstrated the rapid induction of adrenal *Mrap* mRNA by about 5-fold within 2 h in their model of restraint stress, and a 15-fold induction of *Mrap* heteronuclear RNA within 15 min of ACTH injection (although no change in mature *Mrap* mRNA was observed in this time frame). These increases were not sustained and in the restraint stress model *Mrap* mRNA had almost normalized after 4 h. Gibbinson et al. (23) found a 4-fold increase in adrenal *Mrap* mRNA 6 h after LPS injection in the rat (at a time when *Mc2r* mRNA levels had fallen).

Over a longer time period Xing et al. (24) used a microarray approach to explore gene expression changes after 48 h of treatment of human fetal or adult adrenals demonstrating a 16-fold and 12-fold change of *MRAP* and *MC2R* expression (respectively) in adult adrenal, and 8-fold and 11-fold increases in these two genes in fetal adrenal. Hofland et al. (25) demonstrated 11 and 20-fold increases in *MRAP* and *MC2R* in human normal

TABLE 1 | Summary of studies of MC2R and MRAP mRNA expression changes in response to various stimuli.

Species and model	Stimulus	Time	MC2R mRNA	MRAP mRNA	References
Y1 cells	ACTH (10^{-8}M)	24 h	6x	nd	Mountjoy et al. (18)
H-295 cells	ACTH (10^{-8}M)	24 h	2x	nd	Mountjoy et al. (18)
H-295 cells	Forskolin (10^{-5}M)	3 h	1x	nd	Mountjoy et al. (18)
H-295 cells	Forskolin (10^{-5}M)	12 h	3x	nd	Mountjoy et al. (18)
H-295 cells	Forskolin (10^{-5}M)	24 h	3.5x	nd	Mountjoy et al. (18)
H-295 cells	db cAMP (1mM)	24 h	3x	nd	Mountjoy et al. (18)
Human primary cult	ACTH (10^{-8}M)	72 h	21 x	nd	Lebrethon et al. (19)
Human fetal adrenal cells	ACTH (10^{-9}M)	48 h	18 x	nd	Lebrethon et al. (20)
Human fetal adrenal cells	ACTH (10^{-8}M)	3 h	1x	nd	Rehman et al. (21)
Human fetal adrenal cells	ACTH (10^{-8}M)	6 h	3 x	nd	Rehman et al. (21)
Human fetal adrenal cells	ACTH (10^{-8}M)	12 h	12x	nd	Rehman et al. (21)
Human fetal adrenal cells	ACTH (10^{-8}M)	24 h	18 x	nd	Rehman et al. (21)
Rat adrenal (*in vivo*)	Restraint stress	1–4 h	1x	5x (at 2h)	Liu et al. (22)
Rat adrenal (*in vivo*)	ACTH (5μg i.p.)	0–1 h	1x	1x*	Liu et al. (22)
Rat adrenal (*in vivo*)	LPS (25 μg i.v.)	6 h	0.5x	4x	Gibbison et al. (23)
Human fetal adrenal cells	ACTH (10^{-8}M)	48 h	11x	8x	Xing et al. (24)
Human adult adrenal cells	ACTH (10^{-8}M)	48 h	12x	16x	Xing et al. (24)
Human adult adrenal cells	ACTH (10ng/ml)	48 h	20 x	11 x	Hofland et al. (25)
Human adult adrenal cells	Forskolin (10^{-6}M)	48 h	30x	10x	Hofland et al. (25)

**Increase in MRAP hnRNA by 15-fold at 15 mins.*
nd: not measured.

adrenal primary cultures exposed to 48 h treatment with ACTH. These various studies are summarized in **Table 1**.

In summary, although a range of models and stimuli have been used, it is unfortunate that few have examined the response of both *MC2R* and *MRAP* over a time course that encompasses both acute and more delayed time periods. However, it does seem from the available data that while *MC2R* is almost universally induced over a longer time period of 12—24 h or more, *MRAP* is capable of making a more rapid response to ACTH, restraint stress or LPS stimulation.

ACTH RECEPTOR PROTEINS

The next question that arises is what happens to the ACTH receptor protein complex after its translation. Very little data has been collected on the behavior of the MC2R and MRAP proteins—in large part because of absence of good quality antibodies.

It seems that the MRAP homodimer forms at the same time as, or very soon after translation, and that the MRAP molecule in which the N-terminus is in the extracellular position is then glycosylated—probably stabilizing the homodimer at this time. This then interacts with the MC2R protein at the level of the endoplasmic reticulum or Golgi (26). This complex is capable of trafficking to the cell surface, although whether MRAP has an additional role in supporting correct folding of the MC2R is not clear. It is also unclear what the fate of un-complexed MC2R is at the ER—for example in circumstances in which MRAP is not expressed. In studies in cells lacking MRAP, transfected MC2R accumulated substantially at the ER, although the relationship of such overexpression systems to physiology is uncertain (27). It is also clear that in the absence of MC2R, MRAP will traffic efficiently to the cell surface (8, 9). The key elements of this conventional model are shown in **Figure 1**.

ACTH RECEPTOR TURNOVER

In an article published in this journal in 2016, Maben et al traced the fate of MRAP in transfected CHO cells (28). They concluded that MRAP has a half-life of 1.7 h and is not stabilized by the presence of MC2R expression. Using similar techniques they estimated that the MC2R protein half-life was between 3 and 8 h. This is a surprising finding as it implies that after reaching the plasma membrane MRAP and MC2R turn over independently of each other. This is apparently in contradiction to the "lifetime partnership" model and implies that MRAP-independent MC2R is likely to be present at the cell surface under most circumstances and is presumably unresponsive to ACTH.

Clearly this half-life data needs replication although it was obtained using a robust but relatively novel biotin-labeling technique as well as by a more traditional cycloheximide technique with similar findings. It has the disadvantage of deriving from transfected CHO cells rather than cells in which MRAP and MC2R were endogenously expressed. Unquantitated data from transfected OS3 adrenocortical cells was also shown in the same paper which appeared to suggest a substantially longer half life for the MRAP protein—particularly in the absence of MC2R. OS3 cells express endogenous and functional MRAP, but no endogenous MC2R (29). Maben et al. argued that OS3 cells grow more slowly than CHO cells and that this may explain

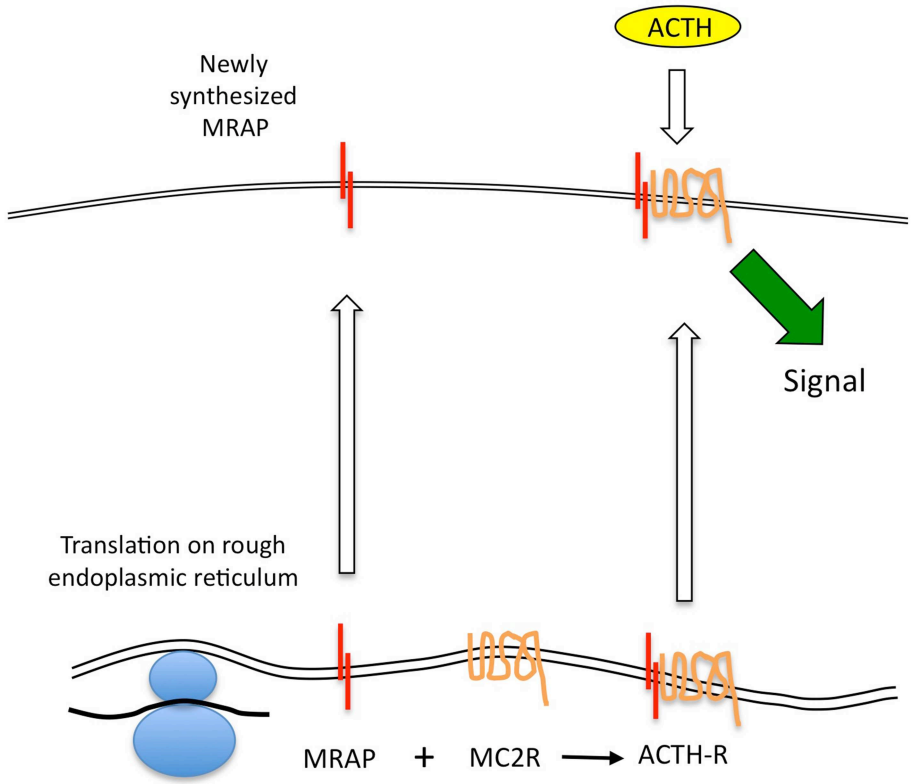

FIGURE 1 | ACTH receptor protein expression. MC2R mRNA is translated at the endoplasmic reticulum (ER), and may independently adopt a correctly folded and orientated receptor conformation. However, it is unable to traffic beyond this point to the plasma membrane. MRAP mRNA is translated and adopts an anti-parallel homodimeric conformation at the ER. This complexes with the MC2R protein and may assist with its correct conformational folding before enabling the complex to traffic to the cell surface. Only the MRAP-MC2R complex is competent to bind ACTH at physiological concentrations and thence to generate a steroidogenic signal.

the slower turnover, but clearly an alternative explanation is that adrenal cells protect the turnover of these proteins for functional reasons. This question needs further exploration.

If we accept that MRAP protein does turnover rapidly and at a faster rate than MC2R, it seems probable that the ACTH receptor complex is not a tight one and that free MRAP (presumably in its homodimeric form) and MC2R may be present at the cell surface. Unfortunately there is no published data on the relative quantities of these two proteins in adrenal cells expressing functional ACTH receptors. There is also no data on the affinities of the MRAP dimer for the receptor. Roy et al provided immunofluorescent imaging data to support the existence of non-complexed MC2R and MRAP in transfected cells (30). If there is free MC2R on the cell surface this would form a reservoir of "spare" receptor that was incapable of responding to ACTH. Consequently, the relatively rapid transcription of *MRAP* in response to the various stimuli described earlier may enable functionally "new" receptor to be recruited *at the cell surface* in the absence of rapid *MC2R* gene expression. This would provide an attractive and efficient mechanism for the rapid upregulation of the ACTH receptor without having to depend on the accurate folding of new MC2R protein. Elements of this model are shown in **Figure 2**.

The ACTH receptor desensitizes and internalizes after agonist stimulation via clathrin-coated pits and re-cycles directly, or via the Golgi-recycling pathway (31, 32). Around 70% of the cell surface receptor that binds ACTH does not internalize however (31, 32) (**Figure 3**), and it would be interesting to know if this is the un-partnered MC2R that was unable to signal. This may be a false conclusion however as Sebag and Hinkle (11) have shown that MRAP determines ACTH ligand binding via a specific LDYI motif in the N-terminal region, so un-partnered MC2R may be unlikely to bind the ACTH tracer used in these studies. This situation may be more complex however in that MRAP2, a paralogue of MRAP, which lacks the critical LDYI ACTH binding motif of MRAP will support ACTH signaling, and hence binding, at high (micromolar) ACTH concentrations (33).

Other than the adrenal cortex, MRAP is also expressed in adipocytes, and indeed was first identified as a protein of unknown function that was expressed as 3T3-L1 fibroblasts differentiated into adipocytes (34). Zhang et al. (35) have shown in differentiated 3T3-L1 adipocytes that the N-terminus of MRAP interacts with the $G\alpha_s$ protein and that the region between residues 14 and 17 of MRAP is critical for this role. While these findings may be compatible with each other, Sebag and Hinkle had previously demonstrated that the MRAP 14-17

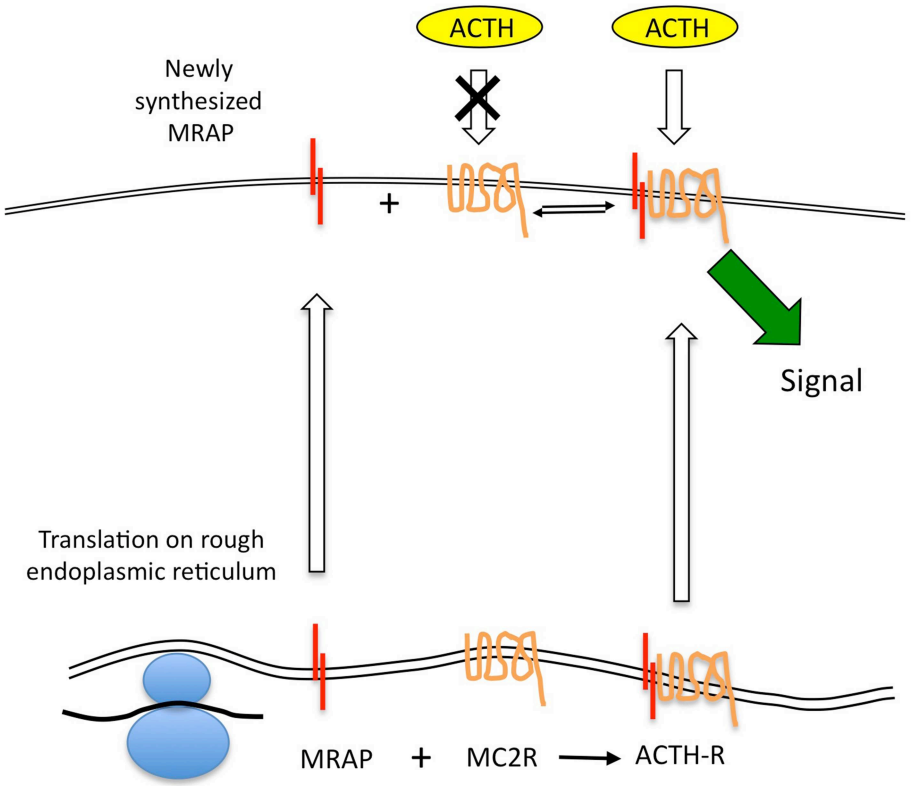

FIGURE 2 | The MRAP-MC2R ACTH receptor complex may be relatively labile allowing MRAP protein to turnover more quickly than the MC2R, and thus leaving uncomplexed MC2R, which is believed to be unresponsive to ACTH stimulation, at the cell surface. This open the possibility that newly transcribed MRAP mRNA can be quickly translated and traffic to the cell surface to complex with "free" MC2R. Such a model permits a rapid response in the form of new signaling-competent receptor recruitment.

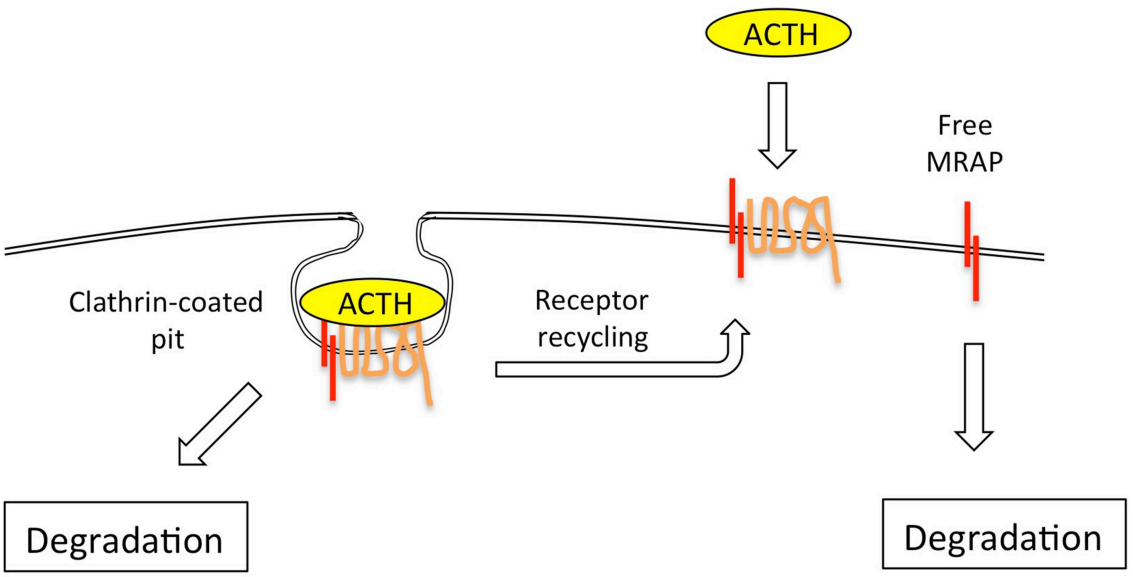

FIGURE 3 | The ACTH receptor complex internalizes via clathrin-coated pits, following which receptor is either rapidly re-cycled to the plasma membrane, or degraded. The evidence suggests that MRAP is also independently and fairly rapidly degraded via ubiquitin-dependent and independent mechanisms.

deletion mutant was fully capable of signaling in their earlier studies (11).

CONCLUSIONS

MRAP is a very unusual protein that appears to have a critical role in permitting the MC2R to traffic to the cell surface and to respond to ACTH—either by influencing ACTH ligand binding or by approximating the $G\alpha_s$ protein to the receptor (or both). ACTH receptor expression can be stimulated by a variety of exogenous stimuli including ACTH exposure. This is mediated by the relatively rapid increase of MRAP transcription while MC2R transcription is more delayed. Newly translated MRAP will enable any MC2R still resident at the endoplasmic reticulum to traffic to the cell surface, but it seems more likely that most of the newly synthesized MRAP will traffic independently to the cell surface where it may partner with un-partnered MC2R, thus creating new functional receptor. Consistent with this is the observation that MRAP protein may turnover more rapidly than MC2R, although the mechanisms and cellular location for this degradation are not clear.

AUTHOR CONTRIBUTIONS

This work is the result of discussion between the authors. The initial draft was prepared by AC and revised by both authors.

FUNDING

LC was supported by this Fellowship from the MRC.

ACKNOWLEDGMENTS

LC was recipient of the Medical Research Council (MRC) UK/Academy of Medical Sciences Clinician Scientist Fellowship Grant G0802796 that supported work reviewed in the manuscript and is currently funded by IFCAH (International Funding for Congenital Adrenal Hyperplasia).

REFERENCES

1. Haynes RC Jr. The activation of adrenal phosphorylase by the adrenocorticotropic hormone. *J Biol Chem*. (1958) 233:1220–2.
2. Lefkowitz RJ, Roth J, Pricer W, Pastan I. ACTH receptors in the adrenal: specific binding of ACTH-125I and its relation to adenyl cyclase. *Proc Natl Acad Sci USA*. (1970) 65:745–52. doi: 10.1073/pnas.65.3.745
3. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science*. (1992) 257:1248–51. doi: 10.1126/science.1325670
4. Clark AJ, McLoughlin L, Grossman A. Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet*. (1993) 341:461–2. doi: 10.1016/0140-6736(93)90208-X
5. Tsigos C, Arai K, Hung W, Chrousos GP. Hereditary isolated glucocorticoid deficiency is associated with abnormalities of the adrenocorticotropin receptor gene. *J Clin Invest*. (1993) 92:2458–61. doi: 10.1172/JCI116853
6. Weber A, Kapas S, Hinson J, Grant DB, Grossman A, Clark AJ. Functional characterization of the cloned human ACTH receptor: impaired responsiveness of a mutant receptor in familial glucocorticoid deficiency. *Biochem Biophys Res Commun*. (1993) 197:172–8. doi: 10.1006/bbrc.1993.2456
7. Yang YK, Ollmann MM, Wilson BD, Dickinson C, Yamada T, Barsh GS, et al. Effects of recombinant agouti-signaling protein on melanocortin action. *Mol Endocrinol*. (1997) 11:274–80. doi: 10.1210/mend.11.3.9898
8. Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, et al. (2005). Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat Genet*. 37:166–70. doi: 10.1038/ng1501
9. Sebag JA, Hinkle PM. Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proc Natl Acad Sci USA*. (2007) 104:20244–49. doi: 10.1073/pnas.0708916105
10. Cooray SN, Almiro Do Vale I, Leung KY, Webb TR., Chapple JP, Egertova M, et al. The melanocortin 2 receptor accessory protein exists as a homodimer and is essential for the function of the melanocortin 2 receptor in the mouse y1 cell line. *Endocrinology*. (2008) 149:1935–41. doi: 10.1210/en.2007-1463
11. Sebag JA, Hinkle PM. Opposite effects of the melanocortin-2 (MC2) receptor accessory protein MRAP on MC2 and MC5 receptor dimerization and trafficking. *J Biol Chem*. (2009) 284:22641–48. doi: 10.1074/jbc.M109.022400
12. Hinkle PM, Serasinghe MN, Jakabowski A, Sebag JA, Wilson K, et al. Use of chimeric melanocortin-2 and−4 receptors to identify regions responsible for ligand specificity and dependence on melanocortin 2 receptor accessory protein. *Eur J Pharmacol*. (2011) 660:94–102. doi: 10.1016/j.ejphar.2010.10.113
13. Novoselova TV, Jackson D, Campbell DC, Clark AJ, Chan LF. Melanocortin receptor accessory proteins in adrenal gland physiology and beyond. *J Endocrinol*. (2013) 217:R1–11. doi: 10.1530/JOE-12-0501
14. Webb TR, Clark AJ. Minireview: the melanocortin 2 receptor accessory proteins. *Mol Endocrinol*. (2010) 24:475–84. doi: 10.1210/me.2009-0283
15. Durand P, Locatelli A. Up regulation of corticotrophin receptors by ACTH1-24 in normal and hypophysectomized rabbits. *Biochem Biophys Res Commun*. (1980) 96:447–56. doi: 10.1016/0006-291X(80)91235-8
16. Durand P, Locatelli A, Cathiard AM, Dazord A, Saez JM. ACTH induction of the maturation of ACTH-sensitive adenylate cyclase system in the ovine fetal adrenal. *J Steroid Biochem*. (1981) 15:445–8. doi: 10.1016/0022-4731(81)90312-5
17. Penhoat A, Jaillard C, Saez JM. Corticotropin positively regulates its own receptors and cAMP response in cultured bovine adrenal cells. *Proc Natl Acad Sci USA*. (1989) 86:4978–4981. doi: 10.1073/pnas.86.13.4978
18. Mountjoy KG, Bird IM, Rainey WE, Cone RD. ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. *Mol Cell Endocrinol*. (1994) 99:R17–20. doi: 10.1016/0303-7207(94)90160-0
19. Lebrethon MC, Naville D, Begeot M, Saez JM. Regulation of corticotropin receptor number and messenger RNA in cultured human adrenocortical cells by corticotropin and angiotensin II. *J Clin Invest*. (1994) 93:1828–33. doi: 10.1172/JCI117168
20. Lebrethon MC, Jaillard C, Naville D, Begeot M, Saez JM. Regulation of corticotropin and steroidogenic enzyme mRNAs in human fetal adrenal cells by corticotropin, angiotensin-II and transforming growth factor beta 1. *Mol Cell Endocrinol*. (1994) 106:137–43. doi: 10.1016/0303-7207(94)90195-3
21. Rehman KS, Sirianni R, Parker CR Jr, Rainey WE, Carr BR. The regulation of adrenocorticotrophic hormone receptor by corticotropin-releasing hormone in human fetal adrenal definitive/transitional zone cells. *Reprod Sci*. (2007) 14:578–87. doi: 10.1177/1933719107307908
22. Liu Y, Smith LI, Huang V, Poon V, Coello A, et al. Transcriptional regulation of episodic glucocorticoid secretion. *Mol Cell Endocrinol*. (2013) 371:62–70. doi: 10.1016/j.mce.2012.10.011
23. Gibbison B, Spiga F, Walker JJ, Russell GM, Stevenson K., Kershaw Y, et al. Dynamic pituitary-adrenal interactions in response to cardiac surgery. *Crit Care Med*. (2015) 43:791–800. doi: 10.1097/CCM.0000000000000773
24. Xing Y, Parker CR, Edwards M, Rainey WE. ACTH is a potent regulator of gene expression in human adrenal cells. *J Mol Endocrinol*. (2010) 45:59–68. doi: 10.1677/JME-10-0006
25. Hofland J, Delhanty PJ, Steenbergen J, Hofland LJ, van Koetsveld PM, van Nederveen FH, et al. Melanocortin 2 receptor-associated protein (MRAP) and MRAP2 in human adrenocortical tissues: regulation of expression and

association with ACTH responsiveness. *J Clin Endocrinol Metab*. (2012) 97:*E*747–54. doi: 10.1210/jc.2011-2328
26. Cooray SN, Chung TT, Mazhar K, Szidonya L, Clark AJ. Bioluminescence resonance energy transfer reveals the adrenocorticotropin (ACTH)-induced conformational change of the activated ACTH receptor complex in living cells. *Endocrinology*. (2011) 152:495–502. doi: 10.1210/en.2010-1053
27. Noon LA, Franklin JM, King PJ, Goulding NJ, Hunyady L, Clark AJ. Failed export of the adrenocorticotrophin receptor from the endoplasmic reticulum in non-adrenal cells: evidence in support of a requirement for a specific adrenal accessory factor. *J Endocrinol*. (2002) 174:17–25. doi: 10.1677/joe.0.1740017
28. Maben ZJ, Malik S, Jiang LH, Hinkle PM. Dual topology of the melanocortin-2 receptor accessory protein is stable. *Front Endocrinol*. (2016) 7:96. doi: 10.3389/fendo.2016.00096
29. Schimmer BP, Kwan WK, Tsao J, Qiu R. Adrenocorticotropin-resistant mutants of the Y1 adrenal cell line fail to express the adrenocorticotropin receptor. *J Cell Physiol*. (1995) 163:164–71. doi: 10.1002/jcp.1041630119
30. Roy S, Rached M, Gallo-Payet N. Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms alpha and beta in isogenic human embryonic kidney 293 cells. *Mol Endocrinol*. (2007) 21:1656–69. doi: 10.1210/me.2007-0041
31. Roy S, Roy SJ, Pinard L, Taillefer LD, Rached M, Parent JL, et al. Mechanisms of melanocortin-2 receptor (MC2R) internalization and recycling in human embryonic kidney (HEK) cells: identification of key Ser/Thr (S/T) amino acids. *Mol Endocrinol*. (2011) 25:1961–77. doi: 10.1210/me.2011-0018
32. Baig AH, Swords FM, Szaszak M, King PJ, Hunyady L, Clark AJ. Agonist activated adrenocorticotropin receptor internalizes via a clathrin-mediated G protein receptor kinase dependent mechanism. *Endocr Res*. (2002) 28:281–9. doi: 10.1081/ERC-120016798
33. Gorrigan RJ, Guasti L, King P, Clark AJ, Chan LF. Localisation of the melanocortin-2-receptor and its accessory proteins in the developing and adult adrenal gland. *J Mol Endocrinol*. (2011) 46:227–32. doi: 10.1530/JME-11-0011
34. Xu A, Choi KL, Wang Y, Permana PA, Xu LY, et al. Identification of novel putative membrane proteins selectively expressed during adipose conversion of 3T3-L1 cells. *Biochem Biophys Res Commun*. (2002) 293:1161–7. doi: 10.1016/S0006-291X(02)00354-6
35. Zhang X, Saarinen AM, Campbell LE, De Filippis EA, Liu J. Regulation of lipolytic response and energy balance by melanocortin 2 receptor accessory protein (MRAP) in adipocytes. *Diabetes*. (2018) 67:222–34. doi: 10.2337/db17-0862

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 06 August 2019
doi: 10.3389/fendo.2019.00519

The Role of β-Arrestin Proteins in Organization of Signaling and Regulation of the AT1 Angiotensin Receptor

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AT1 angiotensin receptor plays important physiological and pathophysiological roles in the cardiovascular system. Renin-angiotensin system represents a target system for drugs acting at different levels. The main effects of ATR1 stimulation involve activation of Gq proteins and subsequent IP3, DAG, and calcium signaling. It has become evident in recent years that besides the well-known G protein pathways, AT1R also activates a parallel signaling pathway through β-arrestins. β-arrestins were originally described as proteins that desensitize G protein-coupled receptors, but they can also mediate receptor internalization and G protein-independent signaling. AT1R is one of the most studied receptors, which was used to unravel the newly recognized β-arrestin-mediated pathways. β-arrestin-mediated signaling has become one of the most studied topics in recent years in molecular pharmacology and the modulation of these pathways of the AT1R might offer new therapeutic opportunities in the near future. In this paper, we review the recent advances in the field of β-arrestin signaling of the AT1R, emphasizing its role in cardiovascular regulation and heart failure.

Keywords: AT1 receptor, angiotensin II, signaling, biased agonism, arrestin

OPEN ACCESS

Edited by:
Eric Reiter,
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *26 April 2019*
Accepted: *15 July 2019*
Published: *06 August 2019*

Citation:
Turu G, Balla A and Hunyady L (2019) The Role of β-Arrestin Proteins in Organization of Signaling and Regulation of the AT1 Angiotensin Receptor. Front. Endocrinol. 10:519. doi: 10.3389/fendo.2019.00519

INTRODUCTION

AT1 angiotensin II receptor (AT1R) belongs to the G protein-coupled receptor (GPCR) family of membrane receptors and is activated by the octapeptide hormone, angiotensin II (AngII). AngII is the main effector of the renin-angiotensin system (RAS), which has pleiotropic effects in the cardiovascular system and salt-water balance regulation. As such, the RAS is an important target in the treatment of various cardiovascular diseases. AT1R blockers (ARBs) and angiotensin-converting enzyme inhibitors are widely used for the treatment of hypertension. Moreover, ARBs have beneficial effects beyond lowering the blood pressure, by preventing cardiovascular organ injuries (1). AngII can bind and activate two types of angiotensin receptors (type 1 and type 2 angiotensin receptors). Some cells contain AT2-receptors that mostly act via Gi and tyrosine phosphatases showing somewhat opposite effects to the AT1R-mediated cellular responses (2). However, the main physiological and pathological effects of RAS are mediated by AT1R. Initially, G protein activation was considered to be the only signal transduction process, which mediates the effects of AngII via AT1R. After AngII binding to AT1R, a heterotrimeric G protein, Gq mediates the hydrolysis of PtdIns(4,5)P2 by phosphoinositide-specific phospholipase Cβ resulting in a generation of second messengers. In addition, AT1R can also couple to Gi/o and G12/13

proteins leading to inhibition of adenylyl cyclase, activation of phospholipase D, Rho-kinase, and regulation of Ca^{2+} channels (3, 4).

Following their activation, GPCRs usually undergo G protein kinase (GRK) dependent phosphorylation on serine and threonine residues on their C-terminal tails. This phosphorylation leads to binding of β-arrestin proteins, which shut down G protein activation and target the receptors toward internalization. During the last two decades it became increasingly evident that, in addition to G proteins, β-arrestins can also mediate signaling events. β-arrestin proteins serve as scaffold proteins, bringing together the players of different protein kinase cascades, and instead of turning it off, they switch the signaling toward different routes (5). Like many other GPCRs, AT1R also binds β-arrestins, and β-arrestins regulate their internalization (6–8) Since the recognition of the role of β-arrestins in AT1R internalization, AT1R served as one of the most studied model for β-arrestin-dependent signaling, which has led to a completely new field in molecular pharmacology with the potential of development of new drugs, which may exploit the possibilities in selective activation of GPCR-activated signaling pathways.

STRUCTURAL REQUIREMENTS FOR INTERNALIZATION AND β-ARRESTIN BINDING TO THE AT1R

The binding of agonists to G protein-coupled receptors initiate the "classical," G protein-mediated pathway, which results in the production of second messenger molecules. Termination of signaling has a key role in the regulation of the kinetics of receptor function. Mechanisms regulating receptor function include several consecutive or parallel processes, such as desensitization, internalization into intracellular vesicles, and degradation of the receptors. Receptor internalization is regulated by phosphorylation by GPCR kinases, which promotes the β-arrestin binding leading to desensitization, internalization and altered signaling of GPCRs (9). The role of a serine-threonine rich domain in the C-terminus of AT1R for angiotensin receptor internalization was first described in the laboratory of Kevin J. Catt (10–12). It was initially discovered with mutational and truncation analysis of AT1R that Thr332-Lys333-Met334-Ser335-Thr336-Leu337-Ser338 amino acids, particularly the Ser335-Thr336-Leu337 motif on the C-terminus of the receptor are required for receptor internalization (10). A large number of serine and threonine amino acids in this region suggested the possible involvement of phosphorylation in its regulation. Indeed, Thr332, Ser335, Thr336, and Ser338 amino acids have been later identified as phosphorylation sites that regulate endocytosis (12–14), and involvement of β-arrestins in receptor internalization was verified (6, 15). The same serine and threonine amino acids were identified as the region critical for stable interaction between AT1R and β-arrestins following AngII stimulation (15, 16). On β-arrestin, two critical lysins (K11 and K12 in β-arrestin2), the phosphate-binding residues, are responsible for the stable interaction (17–22). It turns out, that the interaction between the serine/threonine-rich region and these two lysins (the "stability lock") is responsible for the conformational rearrangement in βarrestin2 protein, leading to recruitment of the members (23, 24) and activation of Erk MAPK cascade (16). Interestingly, not only receptor activation, but also PKC mediated phosphorylation of the inactive, unliganded AT1R alone leads to recruitment of β-arrestin2, receptor internalization and scaffolding of the signaling complex (24).

THE CONCEPT OF BIASED AGONISM

Ligand binding to a plasma membrane receptor can initiate several parallel signal transduction pathways leading to various responses in the cell. Ligands of the plasma membrane receptors were originally classified as agonists and antagonists (or more recently inverse agonists). However, several ligands are capable of selectively initiating one or more of distinct signal transduction pathways coupled to one receptor, which phenomenon has been referred to as "biased agonism" or "functional selectivity." It has been revealed that the biased agonism is an important feature of several members of the GPCR superfamily, and it is proposed that the biased agonists can serve as new therapeutic agents (25). Some of the GPCR ligands can selectively couple the given receptor to the different downstream signaling events, including G protein activation and β-arrestin-mediated signaling, leading to altered signaling patterns compared to the natural agonist. The background of this phenomenon is that the variant ligands cause distinct conformational changes in the structure of the receptor. The different conformations can lead to dissimilar interaction capabilities with G proteins and to other partners. Another layer of complexity is introduced by the recognition of different phosphorylation patterns on the receptor C-terminus. It has been recognized, that different receptors bind arrestins with different affinities, some of which form stable (called class B receptors) whereas others form loose interactions with β-arrestins (called class A receptors) (26). Interaction stability and arrestin activation seem to be determined by specific patterns of phosphorylated serine/threonine amino acids (22, 27). Moreover, different kinases may phosphorylate different residues on the C-terminus, resulting in slightly different outcomes regarding internalization, signaling and activated β-arrestin conformations. This phenomenon has led to the barcode theory. Slightly different active receptor conformations, different cellular expression contexts, or even simultaneous stimulation of other receptors can lead to altered phosphorylation patterns, leading to different conformations of β-arrestins, and altered signaling (28–34). AT1R phosphorylation by GRK2/3 regulates receptor endocytosis, while phosphorylation by GRK5/6 activates Erk1/2 (35). Even PKC phosphorylation can initiate β-arrestin binding and recruitment of the MAPK machinery to the AT1R, although the binding affinity is lower and the active conformation of the β-arrestin is altered (24). These data together suggest, that signaling bias is not restricted to the G-protein-β-arrestin axis, but even β-arrestin dependent signaling may be finely tuned toward distinct outcomes depending on the actual stimulation and cellular context.

Early studies had identified mutant receptors of the highly conserved Asp125Arg126Tyr127 sequence of AT1R (i.e., DRY/AAY mutation), which are not able to couple to G proteins, but still capable to internalize and bind β-arrestins (7, 36). Using this mutant AT1R, it has been demonstrated that AT1R can cause ERK 1/2 phosphorylation or Src activation in the absence of G protein activation (7, 37, 38). Furthermore, a biased AT1R agonist, [Sar1,Ile4,Ile8]-AngII (SII-AngII) was developed, which is a mutated octapeptide angiotensin analog unable to activate Gq-proteins, but still recruits β-arrestins to the AT1R and is able to evoke the internalization of the receptor (39). Thus, it acts as a biased agonist, which selectively activates β-arrestins. This octapeptide further widened the possibilities to investigate G protein-independent mechanisms. It has been shown, that this agonist can also stimulate G protein-independent mechanisms (7). It was proposed that G protein-mediated MAP kinase activation regulates nuclear targets, while the β-arrestin-mediated MAP kinases activation affects the phosphorylation of cytoplasmic proteins (40, 41). Also, compared to G-protein-dependent Erk1/2 activation, β-arrestin-dependent Erk1/2 signaling is slower, reaching its maximum after 10 min and remains active for a prolonged time (40). Later, several new AngII peptide analogs have been developed and characterized with a bias toward G protein or β-arrestin signaling (42–44). The discovery of the biased signaling has led to the recognition that AT1R may develop multiple active conformations, and this has been demonstrated by molecular dynamics simulations (45, 46) and experimentally (47–49). Conformational changes within AT1R may be followed with BRET sensors utilizing a small molecule acceptor (FlAsH molecule). When placed on different places within AT1R, different conformational changes following stimulation with distinct ligands can capture multiple activation states of the receptor (47). Different conformational states after binding of the different ligands have been further demonstrated using single molecular spectroscopy (48) and more recently using double electron-electron resonance spectroscopy in AT1R (49). The developed biased agonists serve now as widely used tools to study the biased agonism of the AT1R, and numerous of their effects on cell responses were published in recent years (50).

SIGNALING PATHWAYS REGULATED BY AT1R THROUGH β-ARRESTIN

β-arrestin-mediated mechanisms can be considered as the second wave of signal transduction events resulting in alternative outcomes. Moreover, the agonist binding of AT1R can lead to direct association with wide spectra of cytoplasmic signaling proteins, such as AT1R-associated protein (ATRAP), SHP-2, JAK2, β-arrestins, and phospholipase Cγ, which explains a plethora of AT1R generated signaling mechanisms, including JAK/STAT pathway and MAPK cascade activation (51–54). The attached proteins can initiate diverse cellular responses from the receptor-arrestin complex and can form multiprotein units, "signalosomes" (55).

One such selectively activated pathway of particular interest is the β-arrestin-dependent signaling. β-arrestin-mediated signaling events include activation of Src tyrosine kinases extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) cascades, Akt and p38 mitogen-activated protein kinases (56–59).

Besides the observation of Erk1/2 activation through β-arrestins, many other cellular functions have been discovered to be regulated through GPCRs in a non-canonical G protein-independent way. These pathways include JNK3, p38, and Akt protein kinase regulation (56), some of which have been also implicated in AT1R signaling (60, 61). The pathways may include very broad signaling routes, including the increased protein synthesis via β-arrestin-mediated Mnk1, eIF4E, and ERK1/2 activation (62). The arrestin-biased ligand, SII-AngII is also able to increase the aldosterone synthesis in adrenocortical zona glomerulosa cells via β-arrestin, in addition to the AngII-induced Gq protein-dependent aldosterone production, which can lead to adverse cardiac remodeling and heart failure progression (63). Several proteomic and genetic studies were carried out to investigate the short and long term effects of the treatment of SII-AngII using both AT1R overexpressing cells and primary cell cultures. It has been demonstrated that the SII-AngII could initiate a robust G protein-independent signaling network, and signaling pathways significantly differ than the AngII induced cellular responses. Kendall et al. demonstrated that the SII-AngII treatment changed the phosphorylation state of numerous downstream proteins, such as protein phosphatase 2A and prostaglandin E synthase 3 (64). The inhibitory phosphorylation of protein phosphatase 2A results in Akt activation and phosphorylation of glycogen synthase kinase 3β, whereas the prostaglandin E synthase 3 activation increases prostaglandin production both in AT1R expressing HEK293 cells and in rat aortic vascular smooth muscle cells (64). It is very astonishing that the SII-AngII-induced phosphorylation patterns have limited overlap with those of AngII-induced phosphorylation (64), suggesting that the β-arrestin activation initiates not only spatially, but also qualitatively different signaling events. Analyzing the β-arrestin-mediated phosphoproteome after SII stimulation of AT1R revealed a plethora of changes in protein phosphorylation, including a huge number of kinases and some phosphatases as well (65). Besides the activation of different kinase cascade signaling pathways, β-arrestin-dependent regulations of ion channels have been also reported following AT1R activation, including TRPV4 (66), TRPC3 (67), and CaV1.2 (68) channels.

ROLE OF THE β-ARRESTIN DEPENDENT AT1R SIGNALING IN CARDIAC FUNCTION AND HEART FAILURE (HF)

Since the AngII is a vasopressor hormone, its production and effects are very important in the development of numerous cardiovascular diseases such as hypertension, atherosclerosis, and cardiac hypertrophy. Indeed, AT1R blockers and inhibitors of AngII production (ACE inhibitors) are now extensively used in the treatment of hypertension and other cardiovascular diseases

(69, 70). Special fields of interest, where RAS plays significant roles are cardiac hypertrophy and heart failure, where AngII seems to contribute to the pathophysiology of these conditions.

Both AT1R-activation of G protein-dependent and G protein-independent mechanisms contribute to the development of cardiac hypertrophy. It was demonstrated that heart-specific overexpression of different AT1R mutants can lead to hypertrophy, moreover, the mutant AT1R, which mediates only G protein-independent signaling mechanisms, caused greater cardiac hypertrophy but less apoptosis and fibrosis than overexpression of wild-type AT1R (71). Contrary, in vascular smooth cells (VSMCs) only the G protein-dependent mechanism of AT1R, mediated by EGF receptor transactivation and Rho kinase activation, seems to be important in AngII-induced hypertrophy (72). However, β-arrestin 1 dependent activation of RhoA has been demonstrated as well, and interaction of AT1R with SHP2 through Y319 residue might be involved in EGF receptor transactivation (73, 74). Interestingly, with wild type AT1Rs, cardiac hypertrophy was reported to be dependent on G protein and metalloprotease activation but did not occur after β-arrestin biased SII-AngII stimulation of AT1Rs (75). According to Aplin et al., the G protein-independent signaling supports the non-hypertrophic proliferation of rat cardiomyocytes (76, 77).

In the development of pathological cardiac hypertrophy, mechanical stress is thought to be one of the most important factors. Parallel with the discoveries of the β-arrestin-dependent signaling through AT1R, AT1R receptor was also found to be involved in stretch-induced signaling pathways in various cell types. First, it has been discovered, that in cardiomyocytes, stretch-induced Erk1/2 activation was dependent on AT1Rs (78–80). Since then, many further papers have been published with observations of mechanical stretch-induced, AT1R-mediated signaling in different cell types, including cardiomyocytes (81–85), vascular smooth muscle cells (86–89), renal podocytes (90), endothelial (91), and epidermal cells (92).

Interestingly, mechanical activation of AT1R caused increased affinity toward β-arrestin biased ligand TRV 120023 (93), suggesting stabilization of a biased active receptor conformation. Moreover, protein kinase C and GRK2, the kinase responsible for phosphorylation and subsequent β-arrestin binding of many GPCRs, has been reported to be activated upon stretch in neonatal rat ventricular myocytes (94). Indeed, hypo-osmotic stretch and mechanical stress induced β-arrestin translocation to the AT1R (88, 95). It turns out that EKR1/2 activation by mechanical stress through AT1R is β-arrestin dependent (95). Stretch-induced β-arrestin-dependent signaling may involve Erk1/2, Akt kinases, EGFR transactivation (92, 95), and Src may also play an important role in this process (96). In line with the very recent developments (see below), mechanically activated β-arrestin signaling may also involve the activation of Gi/o proteins through AT1R (97).

The involvement of AT1R- β-arrestin interplay in cardiac function has become more evident with the discovery of the beneficial effects of biased AT1R ligands on the heart contractility. RAS is typically activated in patients with HF, with increased circulating AngII levels. Anti-RAS drugs, like AT1R blockers and ACE inhibitors, have been long proven beneficial in the treatment of HF. It seems that the pathological actions of AT1R on vasoconstriction and heart remodeling are mediated through Gq-protein activation. On the contrary, studies have suggested that TRV120023 and TRV120027, a β-arrestin biased ligand of AT1R, decrease blood pressure similarly to AT1R blockers, but unlike those drugs, it improves cardiac performance, preserves cardiac stroke volume, decreases systemic vascular resistance, improves cardiac output while preserving renal functions in animal models (44, 98).

Moreover, TRV120027, in combination with furosemide, decreased preload and afterload in dog models of HF, while furosemide-induced natriuresis and diuresis were preserved (98). Infusion of biased AT1R ligand TRV120023 into mice with familial dilated cardiomyopathy increased myosin light-chain phosphorylation and improved cardiac contractility (99). Earlier studies have already shown, that SII-AngII treatment can lead to increased cardiomyocyte inotropy and lusitropy (100), and in line with that, Frank-Starling mechanism of the heart, which describes the volume load-contractility relationship of the heart, has been found to be dependent both on AT1R and β-arrestins (101). Besides Ca^{2+} sensitization, in immature mouse cardiomyocytes, TRV120027 activates CaV 1.2 Ca^{2+} channels as well through Src-family tyrosine kinases and casein kinase 2 in a β-arrestin-dependent manner (102).

After the initial promising results with biased AT1R ligands on the heart function, the first human study was reported in 2013 (103) and in the same year, the phase IIb study was initiated in patients with acute HF, and the results were reported in 2017 (104). Unfortunately, the results of this study did not confirm the beneficial effects of the biased AT1R activation, in fact, in none of the primary endpoints was observed an improved outcome. However, in this study, short time therapy was assessed; the patients were treated for only 48–96 h after within 24 h of initial presentation, and then the outcomes of acute heart failure were investigated. TRV120027 itself is an octapeptide, which has a half-life of only about a few minutes, so it has to be administered intravenously, and this restricts its long-time usability in patients. Therefore, although this study failed to justify the use of biased AT1R ligands in HF failure therapy, long-term treatment may be still beneficial. Indeed, a recent study on dilated cardiomyopathy mouse model showed improved cardiac structure and function after 3 months of TRV120067, another biased AT1R ligand treatment (105). However, TRV120067 is also a peptide, which limits its application in human patients in long-term therapy plans. The use of small molecules having β-arrestin biased activity on AT1R might be beneficial, however, to date, only one such molecule has been reported. Although troglitazone is a small molecule, potentially suitable for long-term treatments, this molecule has a very weak affinity toward AT1R, making it unattractive in *in vivo* experiments (106).

Although these experiments have very promising results, some important aspects of the RAS are often overlooked which may influence the *in vivo* results. It is widely accepted now, that AT1R can form heterodimers with other GPCRs, and the function of the heterodimers might be influenced by the active state of the AT1R. A classic example of interaction is bradykinin

B2 receptor, but there are also other examples of interactions (38, 107–117). In HEK293 cells, SII-AngII inhibits B2 receptor-mediated Gq/11-dependent intracellular calcium influx when AT1R is coexpressed, showing that AT1R-biased ligands effects might be mediated not only by β-arrestins, but also by more complex interactions between different GPCRs (118). Another often overlooked aspect of the biased ligand actions is the presence of the other angiotensin II receptor *in vivo*, the AT2 angiotensin receptor (AT2R). Although the AT2R does not bind β-arrestins upon Ang II stimulation (119), it still may play role in *in vivo* effects of the AT1R biased agonists. SII-AngII, TRV120023 and TRV120027 seem to bind to this receptor as well, but their effects were mediated through AT1R in experiments where AT2 receptors were checked (44, 120, 121). However, to our knowledge, the effects of these ligands have not been thoroughly tested on AT2R.

In any case, with the better understanding of the AT1R conformation upon biased activation (45–49), and the readily availability of bioinformatical tools in drug screening (122), the discovery of high affinity drugs with small molecular weight might not be that far.

IS β-ARRESTIN SIGNALING G PROTEIN-INDEPENDENT?

Originally, β-arrestin-mediated signaling was thought to be independent of G protein activation. In fact, AT1R-activated pathways have been usually divided into G protein-dependent and G protein-independent (e.g., β-arrestin-dependent), since AT1R mutants used in the studies and biased AT1R ligands failed to activate the usual Gq-protein signaling with IP3 and DAG production and consequent Ca^{2+} signal generation. It has challenged the concept of the clean β-arrestin dependent Erk1/2 activation when SII-AngII, the most widely used β-arrestin biased ligand, has been shown to activate Gq and Gi proteins, however with much weaker efficacy then AngII (123). In addition, this Erk1/2 activation was dependent on G protein activation. Similarly, the mechanic stretch-induced activation of Erk1/2 signaling seems to be also Gi-dependent, although in this setup, β-arrestin biased ligands (TRV12023 and TRV12026) did not require Gi coupling (97). However, very recently, using pathway-wide BRET signaling sensors, 14 different AT1R ligands have been tested, including the β-arrestin biased ones, such as TRV120027. Strikingly, all of the tested ligands activated Gi and G12 proteins with some efficacy (124), raising the possibility that some β-arrestin-dependent signaling pathways may utilize one or more G protein-dependent signaling mechanisms. Indeed, another recent study has used CRISPR-Cas9 system to switch off all G proteins, including G12/13 and used PTX to inhibit Gi ("zero functional G" setup) (125). They found, that although β-arrestin still couples to a set of GPCRs, with no functional G proteins, the Erk1/2 could not be activated. On the other hand, CRISPR deletion of β-arrestin1/2 results in various effects on Erk1/2 signaling, depending on the cell line and receptor type, it may be enhanced, inhibited or unchanged (126). These data show that the picture of β-arrestin-mediated signaling is more complex than we originally thought. The cellular background, adaptive mechanisms, β-arrestin's dual role (desensitization and activation of β-arrestin-dependent Erk1/2 signaling) lead to a very complex regulation mechanism where the end-effect on the signaling cascade will be determined on the interplay of these aspects (126). The available data suggest that during β-arrestin-signaling a complex interplay occurs between β-arrestin-signaling and kinase cascades. This model could explain the recent developments of the field. Although β-arrestin sequestrates together with all the components of the cRaf/MEK/Erk1/2 cascade, the activation of the most proximal kinase in the cascade may be kicked on by β-arrestin-independent mechanisms, which might be either a G proteins, or in more physiological settings, one of the classical, growth factor receptors (56, 127). So far, at least three different

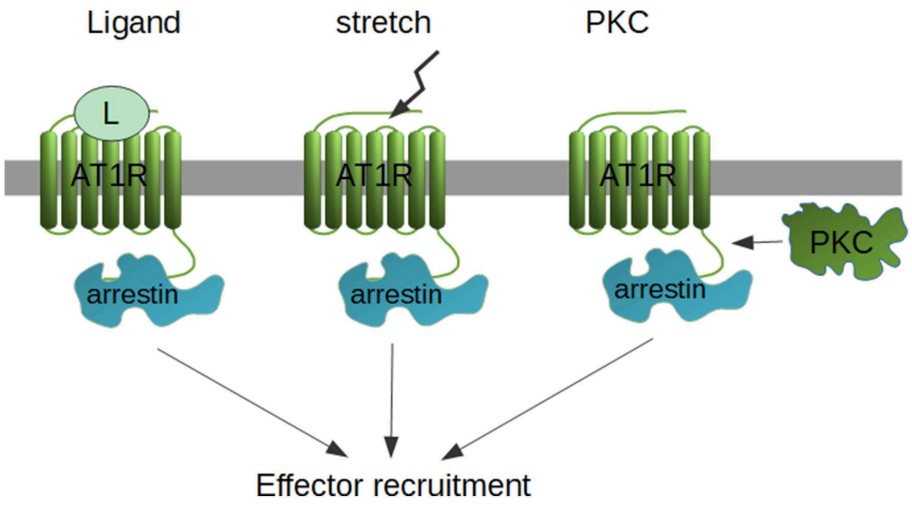

FIGURE 1 | Possibilities of β-arrestin recruitment. β-arrestins are recruited to the AT1R either by activation by ligand or stretch, or by PKC-dependent phosphorylation of the unliganded receptor.

mechanisms may lead to β-arrestin coupling to the AT1R, the classical ligand-dependent way, mechanical stretch and PKC activation (**Figure 1**). When no other stimulus is present, β-arrestins might not be able to activate the most proximal kinases, but when they are already activated, it brings together all the pieces of the signaling cascade and orchestrates their spatiotemporal regulation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We have come a long way in the recent almost 20 years since the interaction of AT1R and β-arrestin has been discovered. The interaction of these two proteins itself had a huge effect in understanding how the GPCRs function, and how are the signaling pathways regulated beside the classical G protein activation. We are at the doorstep of therapeutic exploitation of this system, and although the first attempt to cross it was not successful, recent results suggest that this was probably not our last chance. Recent advances in the field also show that there might be still a lot learn about β-arrestin-dependent signaling, and AT1R might further serve as a good model for such experiments.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by Hungarian National Research, Development and Innovation Fund Grants NVKP_16-1-2016-0039 and K116954.

REFERENCES

1. Ishimitsu T, Honda T, Ohno E, Furukata S, Sudo Y, Nakano N, et al. Year-long antihypertensive therapy with candesartan completely prevents development of cardiovascular organ injuries in spontaneously hypertensive rats. *Int Heart J.* (2010) 51:359–64. doi: 10.1536/ihj.51.359
2. Porrello ER, Delbridge LMD, Thomas WG. The angiotensin II type 2 (AT2) receptor: an enigmatic seven transmembrane receptor. *Front Biosci.* (2009) 14:958–72. doi: 10.2741/3289
3. Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol.* (2006) 20:953–70. doi: 10.1210/me.2004-0536
4. Kawai T, Forrester SJ, O'Brien S, Baggett A, Rizzo V, Eguchi S. AT1 receptor signaling pathways in the cardiovascular system. *Pharmacol Res.* (2017) 125:4–13. doi: 10.1016/j.phrs.2017.05.008
5. Shukla AK, Xiao K, Lefkowitz RJ. Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci.* (2011) 36:457–69. doi: 10.1016/j.tibs.2011.06.003
6. Gáborik Z, Szaszák M, Szidonya L, Balla B, Paku S, Catt KJ, et al. β-arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. *Mol Pharmacol.* (2001) 59:239–47. doi: 10.1124/mol.59.2.239
7. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA.* (2003) 100:10782–7. doi: 10.1073/pnas.1834556100
8. Zhang J, Barak LS, Anborgh PH, Laporte SA, Caron MG, Ferguson SSG. Cellular trafficking of G protein-coupled receptor/β-arrestin endocytic complexes. *J Biol Chem.* (1999) 274:10999–1006. doi: 10.1074/jbc.274.16.10999
9. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. β-arrestins and cell signaling. *Annu Rev Physiol.* (2007) 69:483–510. doi: 10.1146/annurev.physiol.69.022405.154749
10. Hunyady L, Bor M, Balla T, Catt KJ. Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT1 angiotensin receptor. *J Biol Chem.* (1994) 269:31378–82.
11. Hunyady L, Catt KJ, Clark AJL, Gáborik Z. Mechanisms and functions of AT(1) angiotensin receptor internalization. *Regul Pept.* (2000) 91:29–44. doi: 10.1016/S0167-0115(00)00137-3
12. Smith RD, Hunyady L, Olivares-Reyes JA, Mihalik B, Jayadev S, Catt KJ. Agonist-induced phosphorylation of the angiotensin AT1a receptor is localized to a serine/threonine-rich region of its cytoplasmic tail. *Mol Pharmacol.* (1998) 54:935–41. doi: 10.1124/mol.54.6.935
13. Qian H, Pipolo L, Thomas WG. Identification of protein kinase C phosphorylation sites in the angiotensin II (AT1A) receptor. *Biochem J.* (1999) 343(Pt 3):637–44. doi: 10.1042/bj3430637
14. Thomas WG, Motel TJ, Kule CE, Karoor V, Baker KM. Phosphorylation of the angiotensin II (AT 1A) receptor carboxyl terminus: a role in receptor endocytosis. *Mol Endocrinol.* (1998) 12:1513–24. doi: 10.1210/mend.12.10.0179
15. Qian H, Pipolo L, Thomas WG. Association of β-Arrestin 1 with the type 1A angiotensin II receptor involves phosphorylation of the receptor carboxyl terminus and correlates with receptor internalization. *Mol Endocrinol.* (2001) 15:1706–19. doi: 10.1210/me.15.10.1706
16. Wei H, Ahn S, Barnes WG, Lefkowitz RJ. Stable interaction between β-arrestin 2 and angiotensin type 1A receptor is required for β-arrestin 2-mediated activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem.* (2004) 279:48255–61. doi: 10.1074/jbc.M406205200
17. Gimenez LE, Kook S, Vishnivetskiy SA, Ahmed MR, Gurevich EV, Gurevich VV. Role of receptor-attached phosphates in binding of visual and non-visual arrestins to G protein-coupled receptors. *J Biol Chem.* (2012) 287:9028–40. doi: 10.1074/jbc.M111.311803
18. Gimenez LE, Babilon S, Wanka L, Beck-Sickinger AG, Gurevich VV. Mutations in arrestin-3 differentially affect binding to neuropeptide Y receptor subtypes. *Cell Signal.* (2014) 26:1523–31. doi: 10.1016/j.cellsig.2014.03.019
19. Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther.* (2006) 110:465–502. doi: 10.1016/j.pharmthera.2005.09.008
20. Shenoy SK, Lefkowitz RJ. Receptor-specific ubiquitination of β-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *J Biol Chem.* (2005) 280:15315–24. doi: 10.1074/jbc.M412418200
21. Shukla AK, Manglik A, Kruse AC, Xiao K, Reis RI, Tseng W-C, et al. Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature.* (2013) 497:137–41. doi: 10.1038/nature12120
22. Zhou XE, He Y, de Waal PW, Gao X, Kang Y, Van Eps N, et al. Identification of phosphorylation codes for arrestin recruitment by g protein-coupled receptors. *Cell.* (2017) 170:457–469.e13. doi: 10.1016/j.cell.2017.07.002
23. Gulyás G, Tóth JT, Tóth DJ, Kurucz I, Hunyady L, Balla T, et al. Measurement of inositol 1,4,5-trisphosphate in living cells using an improved set of resonance energy transfer-based biosensors. *PLoS ONE.* (2015) 10:e0125601. doi: 10.1371/journal.pone.0125601
24. Tóth AD, Prokop S, Gyombolai P, Várnai P, Balla A, Gurevich VV, et al. Heterologous phosphorylation–induced formation of a stability lock permits

regulation of inactive receptors by β-arrestins. *J Biol Chem.* (2018) 293:876–92. doi: 10.1074/jbc.M117.813139
25. Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of β-arrestin- and G protein-biased agonists. *Trends Mol Med.* (2011) 17:126–39. doi: 10.1016/j.molmed.2010.11.004
26. Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, βArrestin1, and βArrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem.* (2000) 275:17201–10. doi: 10.1074/jbc.M910348199
27. Mayer D, Damberger FF, Samarasimhareddy M, Feldmueller M, Vuckovic Z, Flock T, et al. Distinct G protein-coupled receptor phosphorylation motifs modulate arrestin affinity and activation and global conformation. *Nat Commun.* (2019) 10:1261. doi: 10.1038/s41467-019-09204-y
28. Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J Biol Chem.* (2010) 285:7805–17. doi: 10.1074/jbc.M109.091173
29. Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, et al. Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J Biol Chem.* (2011) 286:11506–18. doi: 10.1074/jbc.M110.154526
30. Jean-Charles P-Y, Kaur S, Shenoy SK. G protein-coupled receptor signaling through β-arrestin-dependent mechanisms. *J Cardiovasc Pharmacol.* (2017) 70:142–58. doi: 10.1097/FJC.0000000000000482
31. Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, et al. Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci Signal.* (2011) 4:ra51. doi: 10.1126/scisignal.2001707
32. Smith JS, Rajagopal S. The β-arrestins: multifunctional regulators of G protein-coupled receptors. *J Biol Chem.* (2016) 291:8969–77. doi: 10.1074/jbc.R115.713313
33. Tobin AB, Butcher AJ, Kong KC. Location, location, location site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci.* (2008) 29:413–20. doi: 10.1016/j.tips.2008.05.006
34. Yang F, Yu X, Liu C, Qu C, Gong Z, Liu H, et al. Phospho-selective mechanisms of arrestin conformations and functions revealed by unnatural amino acid incorporation and 19F-NMR. *Nat Commun.* (2015) 6:8202. doi: 10.1038/ncomms9202
35. Kim J, Ahn S, Ren X-R, Whalen EJ, Reiter E, Wei H, et al. Functional antagonism of different G protein-coupled receptor kinases for β-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci USA.* (2005) 102:1442–7. doi: 10.1073/pnas.0409532102
36. Gáborik Z, Jagadeesh G, Zhang M, Spät A, Catt KJ, Hunyady L. The role of a conserved region of the second intracellular loop in AT1 angiotensin receptor activation and signaling. *Endocrinology.* (2003) 144:2220–8. doi: 10.1210/en.2002-0135
37. Hansen JL, Aplin M, Hansen JT, Christensen GL, Bonde MM, Schneider M, et al. The human angiotensin AT1 receptor supports G protein-independent extracellular signal-regulated kinase 1/2 activation and cellular proliferation. *Eur J Pharmacol.* (2008) 590:255–63. doi: 10.1016/j.ejphar.2008.05.010
38. Karip E, Turu G, Süpeki K, Szidonya L, Hunyady L. Cross-inhibition of angiotensin AT1 receptors supports the concept of receptor oligomerization. *Neurochem Int.* (2007) 51:261–7. doi: 10.1016/j.neuint.2007.05.018
39. Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, et al. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol Pharmacol.* (2002) 61:768–77. doi: 10.1124/mol.61.4.768
40. Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem.* (2004) 279:35518–25. doi: 10.1074/jbc.M405878200
41. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. β-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem.* (2002) 277:9429–36. doi: 10.1074/jbc.M106457200
42. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, et al. Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol.* (2011) 80:367–77. doi: 10.1124/mol.111.072801
43. Strachan RT, Sun J, Rominger DH, Violin JD, Ahn S, Rojas Bie Thomsen A, et al. Divergent transducer-specific molecular efficacies generate biased agonism at a G protein-coupled receptor (GPCR). *J Biol Chem.* (2014) 289:14211–24. doi: 10.1074/jbc.M114.548131
44. Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther.* (2010) 335:572–9. doi: 10.1124/jpet.110.173005
45. Cabana J, Holleran B, Leduc R, Escher E, Guillemette G, Lavigne P. Identification of distinct conformations of the angiotensin-II type 1 receptor associated with the $G_{q/11}$ protein pathway and the β-arrestin pathway using molecular dynamics simulations. *J Biol Chem.* (2015) 290:15835–54. doi: 10.1074/jbc.M114.627356
46. Modestia SM, Malta de Sá M, Auger E, Trossini GHG, Krieger JE, Rangel-Yagui CO. Biased agonist TRV027 determinants in AT1R by molecular dynamics simulations. *J Chem Inf Model.* (2019) 59:797–808. doi: 10.1021/acs.jcim.8b00628
47. Devost D, Sleno R, Pétrin D, Zhang A, Shinjo Y, Okde R, et al. Conformational profiling of the AT1 angiotensin II receptor reflects biased agonism, G protein coupling, and cellular context. *J Biol Chem.* (2017) 292:5443–56. doi: 10.1074/jbc.M116.763854
48. Li W, Xu J, Kou X, Zhao R, Zhou W, Fang X. Single-molecule force spectroscopy study of interactions between angiotensin II type 1 receptor and different biased ligands in living cells. *Anal Bioanal Chem.* (2018) 410:3275–84. doi: 10.1007/s00216-018-0956-3
49. Wingler LM, Elgeti M, Hilger D, Latorraca NR, Lerch MT, Staus DP, et al. Angiotensin analogs with divergent bias stabilize distinct receptor conformations. *Cell.* (2019) 176:468–478.e11. doi: 10.1016/j.cell.2018.12.005
50. Takezako T, Unal H, Karnik SS, Node K. Current topics in angiotensin II type 1 receptor research: focus on inverse agonism, receptor dimerization and biased agonism. *Pharmacol Res.* (2017) 123:40–50. doi: 10.1016/j.phrs.2017.06.013
51. Ali MS, Sayeski PP, Dirksen LB, Hayzer DJ, Marrero MB, Bernstein KE. Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT_1 receptor. *J Biol Chem.* (1997) 272:23382–8. doi: 10.1074/jbc.272.37.23382
52. Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ. Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem.* (1999) 274:17058–62. doi: 10.1074/jbc.274.24.17058
53. Marrero MB, Venema VJ, Ju H, Eaton DC, Venema RC. Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. *Am J Physiol Physiol.* (1998) 275:C1216–23. doi: 10.1152/ajpcell.1998.275.5.C1216
54. Venema RC, Ju H, Venema VJ, Schieffer B, Harp JB, Ling BN, et al. Angiotensin II-induced association of phospholipase Cγ1 with the G-protein-coupled AT1 receptor. *J Biol Chem.* (1998) 273:7703–8. doi: 10.1074/jbc.273.13.7703
55. Kendall RT, Luttrell LM. Diversity in arrestin function. *Cell Mol Life Sci.* (2009) 66:2953–73. doi: 10.1007/s00018-009-0088-1
56. Gurevich VV, Gurevich EV. GPCR signaling regulation: the role of GRKs and arrestins. *Front Pharmacol.* (2019) 10:125. doi: 10.3389/fphar.2019.00125
57. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, et al. -arrestin-dependent formation of β2 adrenergic receptor-Src protein kinase complexes. *Science.* (1999) 283:655–61. doi: 10.1126/science.283.5402.655
58. Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, et al. Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc Natl Acad Sci USA.* (2001) 98:2449–54. doi: 10.1073/pnas.041604898
59. McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, et al. -arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science.* (2000) 290:1574–7. doi: 10.1126/science.290.5496.1574
60. Balakumar P, Jagadeesh G. Structural determinants for binding, activation, and functional selectivity of the angiotensin AT1 receptor. *J Mol Endocrinol.* (2014) 53:R71–92. doi: 10.1530/JME-14-0125

61. Strungs EG, Luttrell LM. Arrestin-dependent activation of ERK and Src family kinases. *Handb Exp Pharmacol.* (2014). 219:225–57. doi: 10.1007/978-3-642-41199-1_12
62. DeWire SM, Kim J, Whalen EJ, Ahn S, Chen M, Lefkowitz RJ. β-Arrestin-mediated signaling regulates protein synthesis. *J Biol Chem.* (2008) 283:10611–20. doi: 10.1074/jbc.M710515200
63. Lymperopoulos A, Rengo G, Zincarelli C, Kim J, Soltys S, Koch WJ. An adrenal -arrestin 1-mediated signaling pathway underlies angiotensin II-induced aldosterone production *in vitro* and *in vivo*. *Proc Natl Acad Sci USA.* (2009) 106:5825–30. doi: 10.1073/pnas.0811706106
64. Kendall RT, Strungs EG, Rachidi SM, Lee M-H, El-Shewy HM, Luttrell DK, et al. The β-arrestin pathway-selective Type 1A angiotensin receptor (AT1A) agonist [Sar1,Ile4,Ile8]angiotensin II regulates a robust G protein-independent signaling network. *J Biol Chem.* (2011) 286:19880. doi: 10.1074/jbc.M111.233080
65. Xiao K, Sun J, Kim J, Rajagopal S, Zhai B, Villén J, et al. Global phosphorylation analysis of β-arrestin-mediated signaling downstream of a seven transmembrane receptor (7TMR). *Proc Natl Acad Sci USA.* (2010) 107:15299–304. doi: 10.1073/pnas.1008461107
66. Shukla AK, Kim J, Ahn S, Xiao K, Shenoy SK, Liedtke W, et al. Arresting a transient receptor potential (TRP) channel. *J Biol Chem.* (2010) 285:30115–25. doi: 10.1074/jbc.M110.141549
67. Liu C-H, Gong Z, Liang Z-L, Liu Z-X, Yang F, Sun Y-J, et al. Arrestin-biased AT1R agonism induces acute catecholamine secretion through TRPC3 coupling. *Nat Commun.* (2017) 8:14335. doi: 10.1038/ncomms14335
68. Hermosilla T, Encina M, Morales D, Moreno C, Conejeros C, Alfaro-Valdés HM, et al. Prolonged AT1R activation induces CaV1.2 channel internalization in rat cardiomyocytes. *Sci Rep.* (2017) 7:10131. doi: 10.1038/s41598-017-10474-z
69. Ferrario CM, Mullick AE. Renin angiotensin aldosterone inhibition in the treatment of cardiovascular disease. *Pharmacol Res.* (2017) 125:57–71. doi: 10.1016/j.phrs.2017.05.020
70. Sayer G, Bhat G. The renin-angiotensin-aldosterone system and heart failure. *Cardiol Clin.* (2014) 32:21–32. doi: 10.1016/j.ccl.2013.09.002
71. Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, et al. Cardiac-specific overexpression of AT1 receptor mutant lacking Gq/Gi coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest.* (2005) 115:3045–56. doi: 10.1172/JCI25330
72. Ohtsu H, Higuchi S, Shirai H, Eguchi K, Suzuki H, Hinoki A, et al. Central role of G_q in the hypertrophic signal transduction of angiotensin II in vascular smooth muscle cells. *Endocrinology.* (2008) 149:3569–75. doi: 10.1210/en.2007-1694
73. Barnes WG, Reiter E, Violin JD, Ren X-R, Milligan G, Lefkowitz RJ. β-arrestin 1 and $G_{\alpha q/11}$ coordinately activate RhoA and stress fiber formation following receptor stimulation. *J Biol Chem.* (2005) 280:8041–50. doi: 10.1074/jbc.M412924200
74. Seta K, Sadoshima J. Phosphorylation of tyrosine 319 of the angiotensin II type 1 receptor mediates angiotensin II-induced trans-activation of the epidermal growth factor receptor. *J Biol Chem.* (2003) 278:9019–26. doi: 10.1074/jbc.M208017200
75. Smith NJ, Chan H-W, Qian H, Bourne AM, Hannan KM, Warner FJ, et al. Determination of the exact molecular requirements for type 1 angiotensin receptor epidermal growth factor receptor transactivation and cardiomyocyte hypertrophy. *Hypertension.* (2011) 57:973–80. doi: 10.1161/HYPERTENSIONAHA.110.166710
76. Aplin M, Christensen GL, Schneider M, Heydorn A, Gammeltoft S, Kjølbye AL, et al. Differential extracellular signal-regulated kinases 1 and 2 activation by the angiotensin type 1 receptor supports distinct phenotypes of cardiac myocytes. *Basic Clin Pharmacol Toxicol.* (2007) 100:296–301. doi: 10.1111/j.1742-7843.2007.00064.x
77. Aplin M, Christensen GL, Schneider M, Heydorn A, Gammeltoft S, Kjølbye AL, et al. The angiotensin type 1 receptor activates extracellular signal-regulated kinases 1 and 2 by G protein-dependent and -independent pathways in cardiac myocytes and langendorff-perfused hearts. *Basic Clin Pharmacol Toxicol.* (2007) 100:289–95. doi: 10.1111/j.1742-7843.2007.00063.x
78. Hunyady L, Turu G. The role of the AT1 angiotensin receptor in cardiac hypertrophy: angiotensin II receptor or stretch sensor? *Trends Endocrinol. Metab.* (2004) 15:405–8. doi: 10.1016/j.tem.2004.09.003
79. Yasuda N, Akazawa H, Qin Y, Zou Y, Komuro I. A novel mechanism of mechanical stress-induced angiotensin II type 1–receptor activation without the involvement of angiotensin II. *Naunyn Schmiedebergs Arch Pharmacol.* (2008) 377:393–9. doi: 10.1007/s00210-007-0215-1
80. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, et al. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol.* (2004) 6:499–506. doi: 10.1038/ncb1137
81. Lin L, Tang C, Xu J, Ye Y, Weng L, Wei W, et al. Mechanical stress triggers cardiomyocyte autophagy through angiotensin II type 1 receptor-mediated p38MAP kinase independently of angiotensin II. *PLoS ONE.* (2014) 9:e89629. doi: 10.1371/journal.pone.0089629
82. Naka T, Sakoda T, Doi T, Akagami T, Tsujino T, Masuyama T, et al. Mechanical stretch induced interleukin-18 (IL-18) expression through Angiotensin subtype 1 receptor (AT1R) and endothelin-1 in cardiomyocytes. *Prep Biochem Biotechnol.* (2008) 38:201–12. doi: 10.1080/10826060701885704
83. Oh Y-B, Gao S, Shah A, Kim JH, Park WH, Kim SH. Endogenous angiotensin II suppresses stretch-induced ANP secretion via AT1 receptor pathway. *Peptides.* (2011) 32:374–81. doi: 10.1016/j.peptides.2010.10.031
84. Saygili E, Rana OR, Meyer C, Gemein C, Andrzejewski MG, Ludwig A, et al. The angiotensin-calcineurin-NFAT pathway mediates stretch-induced up-regulation of matrix metalloproteinases-2/-9 in atrial myocytes. *Basic Res Cardiol.* (2009) 104:435–8. doi: 10.1007/s00395-008-0772-6
85. Wang X, Petrie TG, Liu Y, Liu J, Fujioka H, Zhu X. Parkinson's disease-associated DJ-1 mutations impair mitochondrial dynamics and cause mitochondrial dysfunction. *J Neurochem.* (2012) 121:830–9. doi: 10.1111/j.1471-4159.2012.07734.x
86. Hong K, Zhao G, Hong Z, Sun Z, Yang Y, Clifford PS, et al. Mechanical activation of angiotensin II type 1 receptors causes actin remodelling and myogenic responsiveness in skeletal muscle arterioles. *J Physiol.* (2016) 594:7027–47. doi: 10.1113/JP272834
87. Hong K, Li M, Nourian Z, Meininger GA, Hill MA. Angiotensin II type 1 receptor mechanoactivation involves RGS5 (regulator of G protein signaling 5) in skeletal muscle arteries. *Hypertension.* (2017) 70:1264–72. doi: 10.1161/HYPERTENSIONAHA.117.09757
88. Mederos y Schnitzler M, Storch U, Meibers S, Nurwakagari P, Breit A, Essin K, et al. Gq-coupled receptors as mechanosensors mediating myogenic vasoconstriction. *EMBO J.* (2008) 27:3092–103. doi: 10.1038/emboj.2008.233
89. Schleifenbaum J, Kassmann M, Szijártó IA, Hercule HC, Tano JY, Weinert S, et al. Stretch–activation of angiotensin II Type 1_a receptors contributes to the myogenic response of mouse mesenteric and renal arteries. *Circ Res.* (2014) 115:263–72. doi: 10.1161/CIRCRESAHA.115.302882
90. Miceli I, Burt D, Tarabra E, Camussi G, Perin PC, Gruden G. Stretch reduces nephrin expression via an angiotensin II-AT(1)-dependent mechanism in human podocytes: effect of rosiglitazone. *Am J Physiol Renal Physiol.* (2010) 298:F381–90. doi: 10.1152/ajprenal.90423.2008
91. Ramkhelawon B, Rivas D, Lehoux S. Shear stress activates extracellular signal-regulated kinase 1/2 via the angiotensin II type 1 receptor. *FASEB J.* (2013) 27:3008–16. doi: 10.1096/fj.12-222299
92. Kippenberger S, Loitsch S, Guschel M, Müller J, Knies Y, Kaufmann R, et al. Mechanical stretch stimulates protein kinase B/Akt phosphorylation in epidermal cells via angiotensin II type 1 receptor and epidermal growth factor receptor. *J Biol Chem.* (2005) 280:3060–7. doi: 10.1074/jbc.M409590200
93. Tang W, Strachan RT, Lefkowitz RJ, Rockman HA. Allosteric modulation of β-arrestin-biased angiotensin II type 1 receptor signaling by membrane stretch. *J Biol Chem.* (2014) 289:28271–83. doi: 10.1074/jbc.M114.585067
94. Malhotra R, D'Souza KM, Staron ML, Birukov KG, Bodi I, Akhter SA. $G\alpha_q$-mediated activation of GRK2 by mechanical stretch in cardiac myocytes. *J Biol Chem.* (2010) 285:13748–60. doi: 10.1074/jbc.M110.109272
95. Rakesh K, Yoo B, Kim I-M, Salazar N, Kim K-S, Rockman HA. β-arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. *Sci Signal.* (2010) 3:ra46. doi: 10.1126/scisignal.2000769
96. Wang S, Gong H, Jiang G, Ye Y, Wu J, You J, et al. Src is required for mechanical stretch-induced cardiomyocyte hypertrophy

through angiotensin II type 1 receptor-dependent β-Arrestin2 pathways. *PLoS ONE.* (2014) 9:e92926. doi: 10.1371/journal.pone.0092926
97. Wang J, Hanada K, Gareri C, Rockman HA. Mechanoactivation of the angiotensin II type 1 receptor induces β-arrestin-biased signaling through Gα $_i$ coupling. *J Cell Biochem.* (2018) 119:3586–97. doi: 10.1002/jcb.26552
98. Boerrigter G, Soergel DG, Violin JD, Lark MW, Burnett JC. TRV120027, a novel β-arrestin biased ligand at the angiotensin II type I receptor, unloads the heart and maintains renal function when added to furosemide in experimental heart failure. *Circ Hear Fail.* (2012) 5:627–34. doi: 10.1161/CIRCHEARTFAILURE.112.969220
99. Tarigopula M, Davis RT, Mungai PT, Ryba DM, Wieczorek DF, Cowan CL, et al. Cardiac myosin light chain phosphorylation and inotropic effects of a biased ligand, TRV120023, in a dilated cardiomyopathy model. *Cardiovasc Res.* (2015) 107:226–34. doi: 10.1093/cvr/cvv162
100. Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. β-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci USA.* (2006) 103:16284–9. doi: 10.1073/pnas.0607583103
101. Abraham DM, Davis RT, Warren CM, Mao L, Wolska BM, Solaro RJ, et al. β-Arrestin mediates the Frank-Starling mechanism of cardiac contractility. *Proc Natl Acad Sci USA.* (2016) 113:14426–31. doi: 10.1073/pnas.1609308113
102. Kashihara T, Nakada T, Kojima K, Takeshita T, Yamada M. Angiotensin II activates CaV 1.2 Ca2+ channels through β-arrestin2 and casein kinase 2 in mouse immature cardiomyocytes. *J Physiol.* (2017) 595:4207–25. doi: 10.1113/JP273883
103. Soergel DG, Subach RA, Cowan CL, Violin JD, Lark MW. First clinical experience with TRV027: pharmacokinetics and pharmacodynamics in healthy volunteers. *J Clin Pharmacol.* (2013) 53:892–9. doi: 10.1002/jcph.111
104. Pang PS, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, et al. Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF). *Eur Heart J.* (2017) 38:2364–73. doi: 10.1093/eurheartj/ehx196
105. Ryba DM, Li J, Cowan CL, Russell B, Wolska BM, Solaro RJ. Long-term biased β-arrestin signaling improves cardiac structure and function in dilated cardiomyopathy. *Circulation.* (2017) 135:1056–70. doi: 10.1161/CIRCULATIONAHA.116.024482
106. Tilley DG, Nguyen AD, Rockman HA. Troglitazone stimulates β-arrestin-dependent cardiomyocyte contractility via the angiotensin II type 1A receptor. *Biochem Biophys Res Commun.* (2010) 396:921–6. doi: 10.1016/j.bbrc.2010.05.023
107. AbdAlla S, Lother H, Quitterer U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature.* (2000) 407:94–8. doi: 10.1038/35024095
108. Ayoub MA, Zhang Y, Kelly RS, See HB, Johnstone EKM, McCall EA, et al. Functional interaction between angiotensin II receptor type 1 and chemokine (C-C Motif) receptor 2 with implications for chronic kidney disease. *PLoS ONE.* (2015) 10:e0119803. doi: 10.1371/journal.pone.0119803
109. Barki-Harrington L, Luttrell LM, Rockman HA. Dual inhibition of β-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction *in vivo*. *Circulation.* (2003) 108:1611–8. doi: 10.1161/01.CIR.0000092166.30360.78
110. Bellot M, Galandrin S, Boularan C, Matthies HJ, Despas F, Denis C, et al. Dual agonist occupancy of AT1-R–α2C-AR heterodimers results in atypical Gs-PKA signaling. *Nat Chem Biol.* (2015) 11:271–9. doi: 10.1038/nchembio.1766
111. Goupil E, Fillion D, Clément S, Luo X, Devost D, Sleno R, et al. Angiotensin II type I and prostaglandin F2α receptors cooperatively modulate signaling in vascular smooth muscle cells. *J Biol Chem.* (2015) 290:3137–48. doi: 10.1074/jbc.M114.631119
112. Hansen JL, Theilade J, Haunsø S, Sheikh SP. Oligomerization of wild type and nonfunctional mutant angiotensin II type I receptors inhibits Gαq protein signaling but not ERK activation. *J Biol Chem.* (2004) 279:24108–15. doi: 10.1074/jbc.M400092200
113. Jonas KC, Fanelli F, Huhtaniemi IT, Hanyaloglu AC. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *J Biol Chem.* (2015) 290:3875–92. doi: 10.1074/jbc.M114.622498
114. Nishimura A, Sunggip C, Tozaki-Saitoh H, Shimauchi T, Numaga-Tomita T, Hirano K, et al. Purinergic P2Y $_6$ receptors heterodimerize with angiotensin AT1 receptors to promote angiotensin II–induced hypertension. *Sci Signal.* (2016) 9:ra7. doi: 10.1126/scisignal.aac9187
115. Siddiquee K, Hampton J, McAnally D, May L, Smith L. The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition. *Br J Pharmacol.* (2013) 168:1104–17. doi: 10.1111/j.1476-5381.2012.02192.x
116. Szalai B, Barkai L, Turu G, Szidonya L, Várnai P, Hunyady L. Allosteric interactions within the AT1 angiotensin receptor homodimer: role of the conserved DRY motif. *Biochem Pharmacol.* (2012) 84:477–85. doi: 10.1016/j.bcp.2012.04.014
117. Zha D, Cheng H, Li W, Wu Y, Li X, Zhang L, et al. High glucose instigates tubulointerstitial injury by stimulating hetero-dimerization of adiponectin and angiotensin II receptors. *Biochem Biophys Res Commun.* (2017) 493:840–6. doi: 10.1016/j.bbrc.2017.08.047
118. Wilson PC, Lee M-H, Appleton KM, El-Shewy HM, Morinelli TA, Peterson YK, et al. The arrestin-selective angiotensin AT1 receptor agonist [Sar1,Ile4,Ile8]-AngII negatively regulates bradykinin B2 receptor signaling via AT1-B2 receptor heterodimers. *J Biol Chem.* (2013) 288:18872–84. doi: 10.1074/jbc.M113.472381
119. Turu G, Szidonya L, Gáborik Z, Buday L, Spät A, Clark AJL, et al. Differential β-arrestin binding of AT1 and AT2 angiotensin receptors. *FEBS Lett.* (2006) 580:41–5. doi: 10.1016/j.febslet.2005.11.044
120. Carneiro de Morais CP, Polidoro JZ, Ralph DL, Pessoa TD, Oliveira-Souza M, Barauna VG, et al. Proximal tubule NHE3 activity is inhibited by β-arrestin-biased angiotensin II type 1 receptor signaling. *Am J Physiol Physiol.* (2015) 309:C541–50. doi: 10.1152/ajpcell.00072.2015
121. Feng Y-H, Sun Y, Douglas JG. Gβγ-independent constitutive association of Gαs with SHP-1 and angiotensin II receptor AT2 is essential in AT2-mediated ITIM-independent activation of SHP-1. *Proc Natl Acad Sci USA.* (2002) 99:12049–54. doi: 10.1073/pnas.192404199
122. Lo Y-C, Rensi SE, Torng W, Altman RB. Machine learning in chemoinformatics and drug discovery. *Drug Discov Today.* (2018) 23:1538–46. doi: 10.1016/j.drudis.2018.05.010
123. Saulière A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, et al. Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. *Nat Chem Biol.* (2012) 8:622–30. doi: 10.1038/nchembio.961
124. Namkung Y, LeGouill C, Kumar S, Cao Y, Teixeira LB, Lukasheva V, et al. Functional selectivity profiling of the angiotensin II type 1 receptor using pathway-wide BRET signaling sensors. *Sci Signal.* (2018) 11:eaat1631. doi: 10.1126/scisignal.aat1631
125. Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, et al. Lack of β-arrestin signaling in the absence of active G proteins. *Nat Commun.* (2018) 9:341. doi: 10.1038/s41467-017-02661-3
126. Luttrell LM, Wang J, Plouffe B, Smith JS, Yamani L, Kaur S, et al. Manifold roles of β-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. *Sci Signal.* (2018) 11:eaat7650. doi: 10.1126/scisignal.aat7650
127. Gurevich VV, Gurevich EV. Arrestin-mediated signaling: is there a controversy? *World J Biol Chem.* (2018) 9:25–35. doi: 10.4331/wjbc.v9.i3.25

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 29 August 2019
doi: 10.3389/fendo.2019.00576

Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *12 June 2019*
Accepted: *07 August 2019*
Published: *29 August 2019*

Citation:
Dufau ML and Kavarthapu R (2019) Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive. Front. Endocrinol. 10:576. doi: 10.3389/fendo.2019.00576

Gonadotropin Regulated Testicular Helicase (GRTH/DDX25) is member of the DEAD-box family of RNA helicases present in Leydig and germ cells. GRTH is the only family member regulated by hormones, luteinizing hormone, through androgen action. Male mice with knock-out of the GRTH gene are sterile, lack sperm with arrest at round spermatids. GRTH participates on the nuclear export and transport of specific mRNAs, the structural integrity of Chromatoid Bodies of round spermatids, where mRNAs are processed and stored, and in their transit to polyribosomes, where it may regulate translation of relevant genes. GRTH has a central role in the control of germ cell apoptosis and acts as negative regulator of miRNAs which regulate expression of genes involved in the progress of spermatogenesis. In Leydig cells, GRTH gene transcription is regulated by LH via autocrine actions of androgen/androgen receptor and has regulatory effects in steroidogenesis. In germ cells, androgen actions are indirect via receptors in Sertoli cells. Transgenic mice carrying GRTH 5′ flanking region-GFP permitted to discern regions in the gene which directs its expression upstream, in germ cells, and downstream in Leydig cells, and the androgen-regulated transcription at interstitial (autocrine), and germ cell (paracrine) compartments. Further evidence for paracrine actions of androgen/androgen receptor is their transcriptional induction of Germ Cell Nuclear Factor as requisite up-regulator of GRTH gene transcription in round spermatids, linking androgen action to two relevant germ cell genes essential for the progress of spermatogenesis. A missense mutation of R to H at amino acid 242 of GRTH found in 5.8% of a patient population with azoospermia causes loss of the cytoplasmic phospho-GRTH species with preservation of the non-phospho form in transfected cells. Mice with knock-in of the human mutation, lack sperm due to arrest at round spermatids. This model permits to discern the function

of phospho-GRTH. The GRTH phospho-site resides at a Threonine structurally adjacent to the mutant site found in patients. Molecular modeling of this site elucidated the amino acids that form the GRTH/PKA interphase and provide the basis for drug design for use as male contraceptive.

Keywords: GRTH/DDX25, spermatogenesis, round spermatids, androgen, hormonal regulation

DISCOVERY AND EARLY STUDIES

Gonadotropin regulated testicular RNA helicase (GRTH) was discovered in this laboratory in 1999 (1) as a result of screening for LH/hCG responsive genes using differential displayed analysis of RNAs from cultured rat Leydig cells stimulated by high concentrations of gonadotropin. Our intent at the time was to search for a master switch to gain further insights on gonadotropin-induced desensitization of steroidogenic enzymes previously described in our laboratory (2–4). Following single-strand conformation polymorphism analysis, sequencing of the fragments, and verification by RNA protection a single up-regulated fragment that displayed no similarity to any gene of the DATA bank was obtained. Upon screening a rat Leydig cell library a sequence (1,629 bp) that contained an open reading frame encoding 369 amino acids was revealed (1). The *in vitro* transcribed/translated rat cDNA gave a protein of 43 kDa. A data base search indicated similarity of GRTH sequence with members of the DEAD-box family of RNA helicases. In analyzing further clones, we found a single base pair addition which resulted on an opening reading frame of GRTH that contained 357 additional base pair 5′ to the original cDNA ATG codon. The complete sequence thus contains 1,630 bp and an open reading frame of 483 amino acids (5). Although our study did not provide the key to the desensitization process it unveiled a major advance with the discovery of GRTH/DDX25, a testis specific RNA helicase a novel member of the DEAD-box family of RNA helicases. Although this helicase was found initially in Leydig cells soon it was realized that the major function of GRTH is exerted in the germinal epithelium at post-meiotic stages of spermatogenesis. GRTH to date is the only helicase known to be stimulated by hormones. Its transcription is stimulated by gonadotropin (hCG/LH) induced androgen (A) formation/action through stimulation of cell surface LH receptors in Leydig cells and by paracrine action of androgen through Sertoli cells in germ cells of the rat and mouse testis. During the last 20 years, studies in this laboratory have concentrated in understanding the role of GRTH in spermatogenesis and Leydig cell function, its regulation and role in male reproduction.

CHARACTERIZATION OF GRTH ENZYMATIC ACTIVITY AND ITS LOCALIZATION IN LEYDIG AND GERM CELLS

GRTH/DDX25 was the designated trivial/scientific name assigned to this novel RNA helicase. It contains all 9 conserved motifs of the DEAD (Asp-Glu-Ala-Asp)-box RNA helicase family members (Q, I, Ia, Ib, II III, IV, V, VI) including those involved in RNA binding and ATP interaction (6). GRTH contains ATPase activity and ATP dependent RNA helicase activity (**Figure 1A**). It differs from other members of the family in possessing high intrinsic ATPase activity in absence of RNA, but as other family members its activity is highly enhanced in presence of mRNA, synthetic Pol A and DNA while Poly U, total RNA and tRNA, had only minor effect. The ATPase activity either intrinsic or stimulated has an essential Mg^2 requirement. GRTH unwinds messages bidirectionally, experimentally double strands of RNA/RNA, and RNA/DNA 3′ and 5′ duplexes were unwound by GRTH-GST in presence of ATP. GRTH was cloned from the rat Leydig cell, mouse and human testis cDNA libraries (1). Besides the conserved motifs characteristic of the DEAD-box family GRTH has low amino acid sequence similarity with most other members of the family. Its unique N- and C-terminal sequences and overall structure presumably confers its multiple specialized biochemical functions. GRTH mRNA and protein are predominantly expressed in the male gonad and minor levels of the transcripts are expressed in brain, hypothalamus, pituitary and other tissues and stable cell lines in culture including those of rat anterior pituitary and hypothalamic neurons (1, 7). GRTH displays 93–98% amino acid sequence similarity among rat, mouse and human species. It has 64% amino acid identity with DBP5/DDX19 (human/mouse/yeast) which is ubiquitously expressed, associates with nuclear pore complex (9–11) and is required for RNA poly (A) export function (**Figure 1B**). It displays considerably less similarity (41–42%) to initiation factors of the translation complex, eIF4AI,II/DDX2A,B (12) and only 32–34% to other germ cells DEAD-box RNA helicase proteins including, An3 which is expressed throughout oogenesis, embryogenesis and adult life and colocalizes with nucleoli in *Xenopus laevis* oocytes (13), Ded1p/PL10 (*S. cerevisiae*/mouse) require for translation (14) and Vasa/mVH/DDX4, a DEAD-box helicase family member essential for male germ cell development and oogenesis in Drosophila (15). It is also found in the mammalian male germ cell lineage where in its absence spermatogenesis is blocked at the first meiotic cell division (16).

GRTH SPECIES ITS GENERATION AND POST-TRANSCRIPTIONAL MODIFICATIONS AND THEIR CELLULAR LOCALIZATION

Multiple protein GRTH species were generated through usage of three alternative translation initiation codons from a single

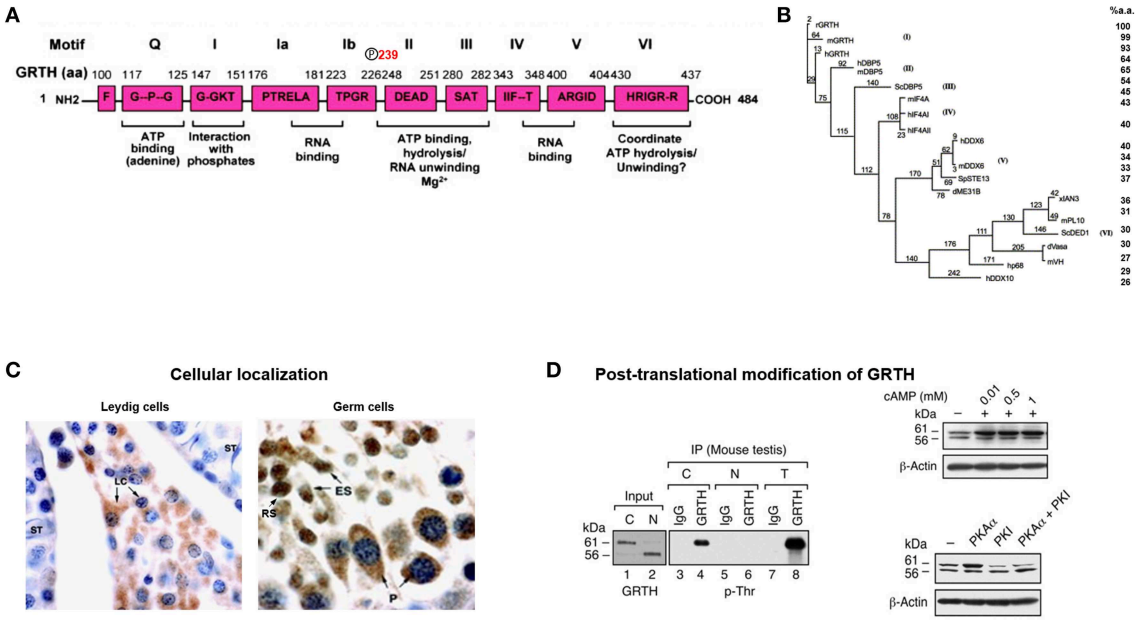

FIGURE 1 | GRTH structure, cellular localization, and post-translational modification. **(A)** Schematic representation of the conserved amino acid motifs shared by GRTH with members of the DEAD-box family of proteins. Nine conserved motifs are indicated as (Q, I, Ia, Ib, II–VI). The amino acid position T239 showing phospho-modification in GRTH which is located outside the conserved domain. **(B)** Phylogenic analysis of members of DEAD-box family of RNA helicase. Maximum parsimony analysis of the alignment of 20 members of DEAD family was constructed using the neighbor-jointing method of PAUP 4.0. The mouse and human GRTH proteins display sequence similarity to the rat of 98.8 and 93.4%, respectively (7). **(C)** Cellular localization in mice testis of GRTH protein in the interstitial compartment and in different cell types in the rat seminiferous tubule (5). GRTH expression is noticed in Leydig cells (LC), pachytene spermatocytes (P), round spermatids (RS), and elongating spermatids (ES). **(D)** Western blot analysis (left) showing pull-down of GRTH protein from mice total testicular lysates (T) and purified cytoplasmic (C) or nuclear (N) fraction using GRTH specific antibody by immunoprecipitation. GRTH; p-Ser, p-Thr, and p-Tyr, phosphoantibodies were used to develop the blot. Right. Western blot showing effect of cAMP treatment on COS-1 cells overexpressing GRTH (above). Western blot showing increased on post-translational modification (phospho) of overexpressed GRTH protein species by co-transfected PKA catalytic subunit (pGRTH, 61 kDa and non-phospho GRTH, 56 kDa) prevented by PKA inhibitor (PKI) (below) in COS-1 cells (8).

transcript of 1.6 kilobases. Testicular germ cells preferentially utilize +1 ATG which closely match the consensus Kozak sequence and yield major proteins of 61/56 kDa. The Leydig cells utilize the 2nd ATG at +343 position resulting in the expression of 48/43 kDa forms. Only in germ cells weak utilization of the 3rd ATG codon at+ 568 producing a 33 kDa protein species was observed (5). ATG codons are utilized in cell specific manner and at least one is hormonal dependent (gonadotropin/cAMP/androgen), since there is switch from 1st to 2nd ATG utilization upon *in vitro* hCG stimulation which was reversed by androgen antagonist Flutamide (5). This was observed in round spermatids not in pachytene spermatocytes. The 2nd ATG is utilized in Leydig cells where endogenous androgen production is abundant and in round spermatids under exogenous hCG treatment which increases androgen levels/actions in the seminiferous tubule (Sertoli cells). Thus, we conclude that utilization of the 2nd ATG is dependent on androgen induced factor(s). This concept is reinforced by more recent findings which demonstrated that the GRTH in round spermatids is transcriptional up-regulated by androgen while pachytene spermatocytes showed no response (17).

The 56 kDa GRTH is the non-phosphorylated form present in the nucleus of germ cells which transports specific nascent messages to the cytoplasm via Chromosomal Maintenance 1 (CRM1) pathway (**Figure 1D**). It is also present in small quantities in the cytoplasm and at this site the function of these species has not been explored. The leucine-rich region at the N-terminal comprising amino acids 61–74, was identified as the nuclear export signal which participates in CRM1-dependent nuclear export pathway of relevant mRNAs to cytosolic sites. Moreover, deletion analysis has localized the GRTH nuclear localization sequence to amino acid positions 100–114, 5′ adjacent to the second ATG codon. The 61 kDa GRTH phospho-species is present solely in the cytoplasm of germ cells [**Figure 1D**; (8)]. This GRTH form by association with relevant messages prevents their degradation and presumably participates in the transport of mRNA to Chromatoid Bodies (CB) of round spermatids for storage prior to translation at specific times during spermatogenesis. The 61 kDa phospho-form also participates in the transport of messages to actively translating polyribosomes where it is believed to engage in the translational regulation of germ cell specific genes involved in the progress of spermatogenesis. Phosphorylation of GRTH is induced by cAMP dependent Protein Kinase A Cα at Threonine 239 [**Figure 1D**; (8, 18)]. This single site resides in the core region, not within any of the conserved motifs of the family. Its

PKA site sequence TKIR is a non-canonical motif which is more common in the testis than in other tissue (19). In the case of 48/43 kDa species it is proposed to represent the phospho-/non-phospho species, respectively, however their specific function need to be established. Both lower molecular forms (48/43 kDa) lack the 5′ sequences where the known nuclear import and export localization sequences for the 61/56 kDa are present, and its intrinsic localization sequences if present remain to be defined. With our present knowledge the 43 kDa form is not expected to reside in the nucleus or engaged in transport of messages, but perhaps its actions are confined to translational process of mRNAs.

CELLULAR DEVELOPMENTAL EXPRESSION OF GRTH IN THE TESTIS

Initial localization of GRTH by *in situ* hybridization in the rat revealed the presence of GRTH transcripts in interstitial and germ cells of the testis. Immuno-labeling was observed within the seminiferous tubules of adult rats in meiotic cells, pachytene spermatocytes and haploid round and elongated spermatids. In contrast, germ cells within the basal compartment (spermatogonia and preleptotene spermatocytes) and Sertoli cells were negative. In pubertal cells only pachytene spermatocytes were positive (1). GRTH mRNA expression was not observed in testis of immature rats, 8–12 days old, while positive signals were observed in tubules and Leydig cells of pubertal animals (ages 23 and 26 days) and in the adult testis. Although there are currently no studies on GRTH expression in neonatal animals, at times when testosterone and LH levels are known to be elevated, it is likely that GRTH expression in neonatal Leydig cells could be temporarily elevated. Northern blot analysis detected a single transcript of 1.6 kb in Leydig and germ cells of the testis and small abundance of transcripts in the hypothalamus, pituitary, and brain but was not found in ovary or other tissues examined including uterus, liver, kidney, heart and adrenal. Also, equivalent size transcripts were detected in the adult human and mouse testis (1). Early *in vitro* studies using recombinant GRTH-GST showed 100–200% increased translation of luciferase RNA template over control indicating the potential role of GRTH in translation. The developmental and stage specific expression of GRTH mRNA pointed to a Gonadotropin/androgen dependence and a participation of this helicase in spermatogenesis. *In vivo* stimulation by hCG of GRTH mRNA in Leydig cells were also observed *in vitro* and similar effects were found upon stimulation of cells with cAMP (8-bromo cAMP) *in vitro*, and an equivalent stimulation of transcripts was induced upon incubation with dihydrotestosterone. Increases induced by hCG and cAMP were prevented by inhibitor of steroidogenic enzymes (cholesterol side chain cleavage, 3β-hydroxysteroid oxidoreductase and 17α-hydroxylase/17-20 desmolase) which abolished the androgen production induced by hCG/cAMP indicating its androgen dependence in Leydig cells (8).

The development of an affinity purified GRTH peptide antibody (amino acid positions 465–467) identified GRTH as a developmentally regulated protein in Leydig and germ cells. Initial immunohistochemistry studies in the rat showed GRTH immunoreactive protein in the interstitial cells of the testis (8). In the seminiferous tubules both pachytene spermatocytes and round spermatids expressed GRTH protein and the intensity of staining varied at different stages of the spermatogenic cycle (**Figure 1C**). Staining in round spermatids attained maximal levels at stages VIII and IX and were reduced at later stages X-XIII of elongated spermatids. In pachytene spermatocytes GRTH protein levels were generally lower than in round spermatids gradually increasing from stages II to IX, and like round spermatids reached peak levels at stages VIII and IX declining thereafter at stages X through XIII. Intense protein staining was observed in stage XIV in spermatocytes entering the metaphase of meiotic division (there are 14 stages of the spermatogenic cycle in the rat and 12 stages in the mouse). In the mouse, peak levels of GRTH protein labeling is observed in round spermatids, pachytene spermatocytes, and elongated spermatids at stages VIII, IX, and X, respectively, and at stage XII is found in spermatocytes entering the metaphase of meiosis. GRTH protein was not found in myoid and Sertoli cells. Combined use of immunocytochemistry studies and western blot analysis demonstrated that GRTH is a developmentally regulated protein in Leydig and germ cells of the rat and mouse testis (8).

GENE STRUCTURE, CELL SPECIFIC EXPRESSION, AND MECHANISMS OF REGULATION BY ANDROGEN IN LEYDIG AND GERM CELLS

The GRTH/DDX25 gene resides in chromosomes 11q24 in human, 8q25 in rat and 9A3-A5 in mouse. The 20 KB mouse gene contains 12 coding exons, all but one of the conserved motifs which is shared between two exons 10 and 11 (motif V), reside within single exons and identical genomic organization was identified of the human GRTH gene. The GRTH gene promoter is TATA-less contains GC sequences, initiator elements, and multiple TSSs. The promoter resides within −205/+63 bp of the gene. The basal transcriptional activity is driven by essential Sp1/Sp3 binding sequences at −169/−150 within the promoter (20). GRTH is regulated by LH through Androgen/Androgen Receptor (AR) directly at the transcriptional level in Leydig cells with impact solely in gonadotropin stimulated steroidogenesis and testosterone production *in vivo* and *in vitro* with no impact in basal conditions. In germ cells however, the action of A is indirect through AR in Sertoli cells and its expression is both cell and stage specific. Sertoli cells are the nursing cells of germ cells with established cellular and molecular communications, thus A actions presumably proceed in a paracrine fashion through signaling events. Generation in our laboratory of transgenic mice models carrying GRTH 5′ flanking regions with GFP as reporter provided *in vivo* systems that permitted differential elucidation of regions that contain sequences in the GRTH gene that directs expression to different cellular compartments, upstream sequences (−6.4/−3.6 kb linked to its promoter −205

to+63-GFP) in germ cells and downstream (−1,085/+63 GFP) in Leydig cells (**Figure 2**). Androgen produced in Leydig cells under the pulsatile LH stimulus from the pituitary gland, stimulates transcription of the GRTH gene in an autocrine manner through its AR association with a non-canonical androgen response element half-site at −827/−822 (5′-TGTCC-3′). This occurs through an association of A/AR bound to its cognate DNA site with member(s) of the preinitiation complex, TFIIB and Pol II via a short-range chromosomal loop revealed by CHIP3C assays. Association of SRC-1 and Med-1 co-activator to A/AR, though not require for looping are essential for AR, TFIIB, and Pol II recruitment to the complex and GRTH transcriptional activation (21). Other studies demonstrated that in transgenic mice the −6.4 kb/+63 GFP transgene directed GRTH expression to both Leydig cells and germ cells (**Figure 2**). Androgen regulation of GRTH transcription *in vivo* and *in vitro* in the germ cell compartment is only confined to round spermatids. The expression of GRTH by androgen is regulated by androgen/AR acting in Sertoli cell which in a paracrine fashion regulate GRTH transcription through a yet to be identified signal(s) relayed to germ cells. This is accomplished by the participation of the transcription factor GCNF (Germ Cell Nuclear Factor) whose expression is regulated by androgen in round spermatids. In these cells GCNF associates to a consensus half-site (−5,270/5,252 nt) of the GRTH gene and promotes its cell specific regulated transcription/expression of this helicase. Moreover, GRTH has been found to be associated with GCNF mRNA and to have an inhibitory effect on GCNF message stability which demonstrate an autocrine regulation of GCNF by GRTH at the post-transcriptional level (22).

LESSONS LEARNT ON THE FUNCTIONS OF GRTH FROM THE GRTH-TARGETED NULL MICE

Phenotype, Functional, and Structural Alterations

GRTH knock-out mice (homozygous) are sterile due to a blockade of spermatogenesis at step 8 of round spermatids resulting in complete lack of elongated spermatids and sperm and solely degenerating spermatids were present in the lumen of the epididymis (**Figures 3A,D**). These mice exhibit normal sexual behavior and have normal circulating levels of gonadotropins and testosterone. This excluded androgen deficiency as the cause of the spermatogenic arrest, despite the existence of EM abnormalities in the Leydig cells (23). These included marked reduction of lipid droplets, swollen mitochondria and lack of typical normal cristae structure while the circulating basal androgen levels were found to be normal in GRTH$^{-/-}$ mice. However, subsequent studies revealed considerable testosterone increase upon stimulation of Leydig cells with high doses of hCG *in vivo* and *in vitro* in null mice when compared to WT. Changes in Leydig cell structure and function are in concert with marked accumulation of cholesterol in the inner mitochondrial membrane and an increase in protein expression

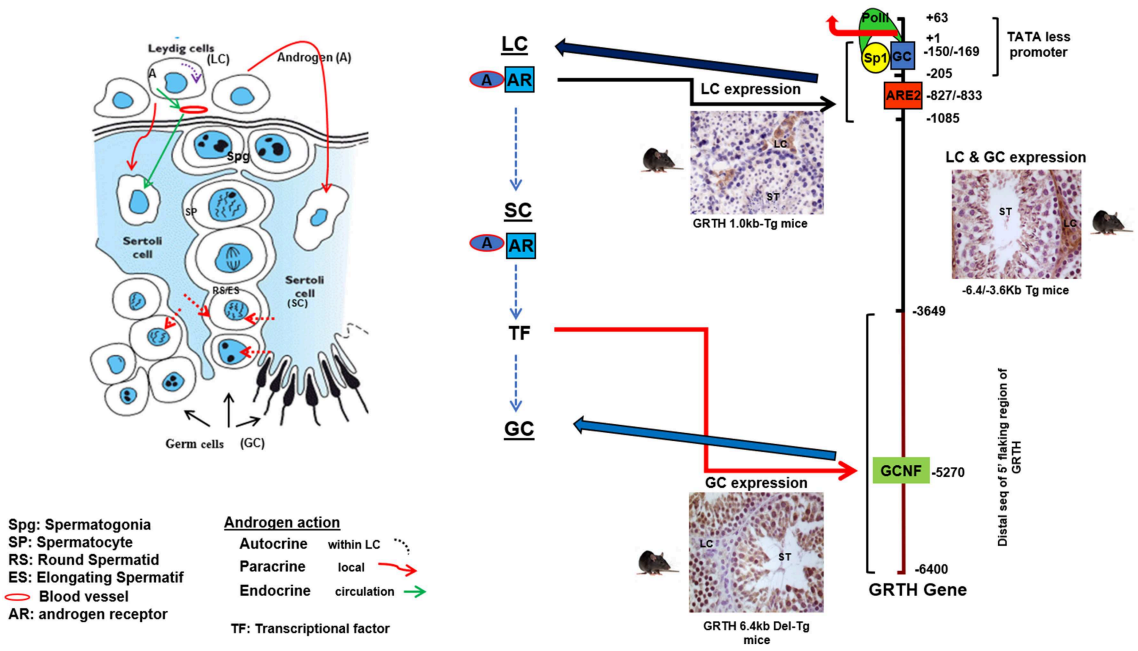

FIGURE 2 | Androgen (A) regulated GRTH expression in cell compartments. Diagram elucidating the 5′-flanking sequence of GRTH gene that directs its cell-specific expression in testicular cells and direct/indirect actions of androgen on GRTH transcription at cellular compartments. A produced from LCs binds to AR and subsequently interacts with ARE2 (−828/−833) to direct GRTH gene expression in LCs. Paracrine activation (yet to be identify) by androgen from LC induces A/AR-responsive gene(s) expression in Sertoli cells. These in turn may activate downstream targets that directly or indirectly induce GRTH transcription through GCNF binding to 5′ elements located in the distal (−3,600/−6,450 bp) 5′-flanking region of the gene (17, 21, 22).

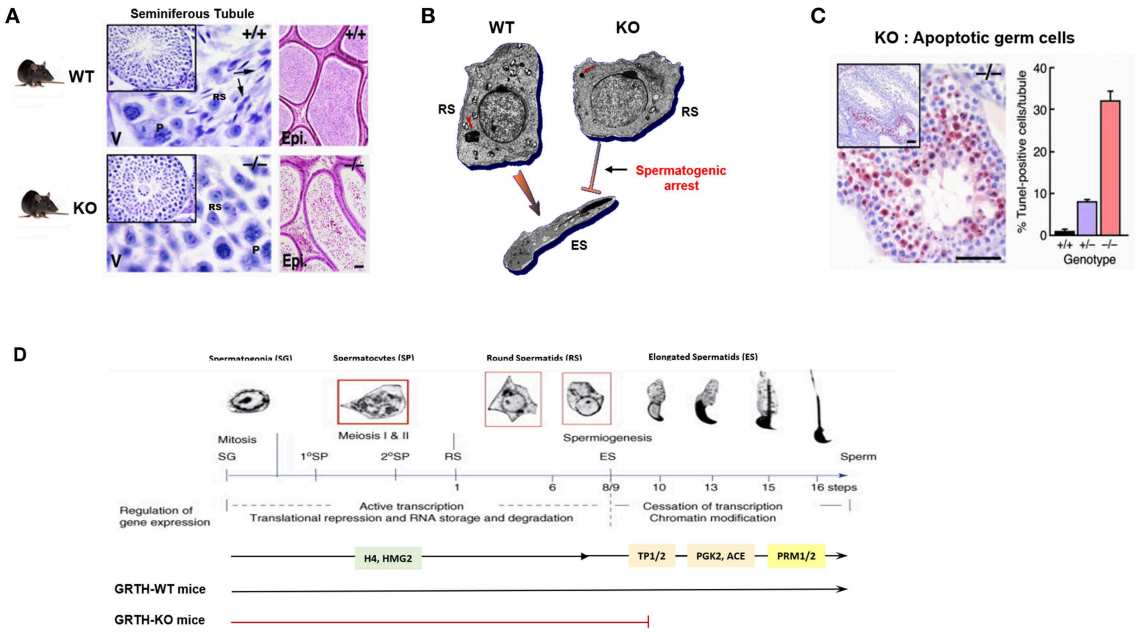

FIGURE 3 | GRTH-KO mice model reveal the role of GRTH in the completion of spermatogenesis. **(A)** H&E staining of WT and KO mice testis showing seminiferous tubules and epididymis. Presence of elongated spermatids only in WT mice is indicated by arrows. P. pachytene spermatocytes; RS, round spermatocytes. Epididymis lumens of WT mice contains mature sperm while in KO mice lumens are filled with degenerating germ cells (23). **(B)** EM images of round spermatid from WT and GRTH-KO mice and elongated spermatid of WT mice. Absence of GRTH in KO mice causes arrest of spermiogenesis at step 8 of round spermatids and failure to elongate (23). CB of KO mice was highly condensed, greatly reduced in size and lacking the typical nuage texture (red arrow) as compared with wild type CB (red arrow). **(C)** TUNEL IHC staining of testis section from GRTH-KO mice show marked apoptosis in germ cells compared to WT mice. Quantitative evaluation of apoptosis (right), mean ± SE of apoptotic cells per tubule [10 tubules were assessed for each group (23)]. **(D)** Diagram of mice spermatogenesis showing spermatogonia (SG, 2n) followed by first meiotic (I) division resulting in the formation of primary (1°) and secondary spermatocytes (SP). Secondary SP through meiosis generate haploid round spermatids (RS) entering the differentiation process of spermiogenesis (16 steps in mouse) to produce elongating spermatids (ES) and spermatozoa/mature sperm. Germ cells expressing GRTH (spermatocytes and round spermatids) are boxed in red. The regulation of gene expression during the developmental process is governed in a precise temporal sequence: an initial active transcription phase with translational repression is followed by cessation of transcription associated with chromatin modification. Chromatin structure in spermatogenic cells changes during development from mitotic spermatogonia into meiotic SP and post-meiotic haploid spermatids. During spermatid elongation, histones are removed and replaced by TP1 and TP2 and subsequently by Prm1 and Prm2. GRTH- dependent expression of proteins is highlighted including H4 (testis specified histone), HMG2, TP2/TP1, testicular ACE, PGK2, Prm1/Prm2. RS development in the WT showing progression to mature sperm. In GRTH-KO, there is a spermatogenic arrest observed at step 8 of RS (7).

of genes involved in cholesterol synthesis and transport including HMGCR, Srebp2, and StAR (24). In contrast, heterozygous mice are fertile. Females homozygous have normal fertility consistent with GRTH expression solely in the male gonad.

Of major note was the highly condensed chromatoid bodies (CB) of markedly reduced size with lack of the usual "nuage" appearance at all steps of round spermatids in GRTH$^{-/-}$ when compared to GRTH $^{+/+}$ and GRTH $^{+/-}$ mice (**Figure 3B**). These changes in the CB of null mice are consistent with their lack of GRTH dependent nuclear-cytoplasmic transport of messages concern with the progress of spermatogenesis (5, 8). The CB is a non-membranous filamentous cytoplasmic body which resides in the cytoplasm adjacent to the nucleus of round spermatids and serve as repository of long-lived mRNAs associated as mRNPs waiting for translation during spermatogenesis. CB contains members of the small interfering RNA pathway like MIWI, argonaute protein, Risc/Dicer endonuclease, decapping enzyme, and mi/si/pi RNAs that participate in the RNA-mediated silencing/degradation (25). CB also contain MVH/DDX4, a mouse homolog of drosophila VASA, which is commonly used as a germ cell marker (15, 26) and proposed to participate in the small RNA interfering pathway to regulate RNA processing (25). MVH has an essential role in the MILI, MIWI, and MIWI2 dependent piRNA processing pathway (27). It is of note that GRTH is highly abundant in the CB (28, 29). In elongating spermatids, the CB moves to a caudal site at the base of the flagellum, where it subsequently undergoes fragmentation and disappears from the cytoplasm (30). There is limited information about the specific mechanisms of the CB function during spermatid elongation. In late pachytene spermatocytes a series of small granules which associated with the nuclear envelope and reside between small vesicles are believed to be precursor of CBs (31). More recent studies have indicated that piRNAs originated in pachytene spermatocytes from non-transposon intergenic regions cause massive abrogation of cellular programs in elongated spermatids in preparation of sperm production (32). CB are believed to possess functional similarities to P-bodies and stress granules which contain aggregates of translationally repressed mRNPs associated with the translation repression and mRNA decay machinery in early development in neurons (33, 34). P-bodies contain members of mRNA decapping machinery, including the decapping enzymes

TABLE 1 | Summary - GRTH, a multi-functional protein regulating several cellular events.

Cellular function/Event	Role of GRTH
Apoptosis	GRTH prevents germ cell apoptosis by regulating expression of several genes pro-apoptotic/anti-apoptotic genes (Cas3, Cas8, Cas9, PARP, Bid, Bad, Bak, p53, Bcl2, Bcl-xL, IκBα/β, TRADD) in the Caspase, NFκB, and TNFα/TNF-R1 mediated pathways.
RNA Transport	Participates in nuclear export of germ cell specific mRNAs (Tp2, Prm2, PGK2, tACE) from nucleus to cytoplasmic sites during spermatogenesis
RNA Processing	GRTH as a helicase protein helps in RNA unwinding. It selectively binds to germ cell specific mRNAs and polyribosomes for active translation process during spermatogenesis
RNA Degradation	GRTH prevents degradation of Tp2, Prm2, and Tssk6 mRNA's essential for spermiogenesis
miRNA Regulation	GRTH plays an important role in the negative regulation of testis specific miRNAs (miR469, miR34c, miR470) and other miRNAs like Let7 family members through regulating their biogenesis via Drosha/DGCR8 microprocessor complex
Spermatogenesis	GRTH is essential for spermatid development and completion of spermatogenesis (round spermatid to elongating spermatid). Maintains the integrity of chromatoid body which stores germ cell specific RNAs during spermatogenesis.

and its activators. Stress granules share some components in common with P-bodies, typically contain translation initiation factors eIF4E, eIF4G, eIF4A, eIF4B, poly-A binding protein, eIF3, eIF2, and the 40S ribosomal subunit (25). Further, the CB also resembles the recently described TIS associated granules and the interconnections proposed between TIS granules and ER (TIGER domain structure) could apply to the CB (35). In germ cells/round spermatids, mRNAs are transported from nucleus to the cytoplasm by GRTH via CRM1 pathway where messages are temporarily stored and translationally repressed in the CB awaiting translation during spermiogenesis and where can also undergo degradation. Disruption of GRTH gene did not show changes on steady state mRNA levels of chromatin remodeling gene transcripts and other of relevance to the progress of spermatogenesis including Tp1, Tp2, tACE. This was observed on the background of reduced cytoplasmic levels, which was attributed to their impaired nuclear export. However, their protein was absent in the null mice indicating the post-transcriptional function of GRTH (**Table 1**) (7). Because in KO mice the arrest occurs prior to the elongation where chromatin remodeling proteins are normally expressed in elongated spermatids, it is difficult to assess the relevance of GRTH in the posttranscriptional event but given their association with GRTH in transport and presumably at cytosolic sites it is strongly indicated the prevalent post-transcriptional function of this helicase (8).

GRTH in microRNA Regulation

A set of primary microRNAs were up-regulated in round spermatids of GRTH KO mice. These include testis specific miR-469, testis preferred miR-34c and miR-470 and others such let-7 family members (let-7a/d, b and e-g) and miR203 (**Table 1**). Also, the enzyme complex (Drosha-DGCR8) required to process the Pri-miRNA transcripts to Pre-miRNAs in the nucleus is upregulated in the KO mice This occurs prior to their transport via exportin 5 to the cytoplasm and final processing by Dicer dependent pathways to mature miRNA with residence in the CB. Our studies have identified TP2 and Prm2 as target genes for miR-469. Through binding to the coding regions of the mRNAs of these chromatin remodelers miR-469 represses their protein expression. GRTH has an essential role in the negative regulation of a subset of miRNAs through maintaining their biogenesis via Drosha/DGCR8 microprocessor complex to generate mature miR 469 and others could play a role during spermatogenesis. Control of the temporal progression of spermatogenesis via miR-469 inhibitory action on TP2/Prm2 mRNA at the CB site could be essential for their timely expression for chromatin compaction in spermatids and the progress of spermatogenesis (36).

Role in Apoptosis

In GRTH null mice, major apoptosis was predominant at stage XII of spermatogenesis and confined to spermatocytes entering the metaphase of meiosis, well before the arrest point (step 8 of round spermatids) and subsequently magnified at the various steps of round spermatids (23). This indicated that at the early stage, the development of germ cells were compromised and progression from a reduced number of cells followed. Spermatogenesis progression to haploid steps of round spermatids in the $GRTH^{-/-}$ mice where apoptosis was so prevalent in pachytene spermatocytes (30% cells/tubule), indicated the participation of yet to be defined compensatory mechanism(s) during these important developmental stages of spermatogenesis (**Figure 3C**). These could include activation of survival mechanisms, noted by the increase of DNA repair proteins (Rad 51 and Dmc1) required for maintaining chromosomal integrity in GRTH null mice and the possible participation of other helicases such as DDX3 and DBP5 in the progression from the remaining viable meiotic cells to haploid steps of round spermatids until the arrest point at step 8. The enhanced apoptosis in the GRTH null mice is undoubtedly related to the absence of GRTH protein since apoptotic cells in spermatocytes were only reduced to 8% per tubule in $GRTH^{+/-}$ mice and <1% in wild type mice. Comparative studies in pachytene spermatocytes of GRTH null mice vs. Wild type revealed that Pro- and anti-apoptotic factors are regulated by GRTH. Significant increased levels of pro-apoptotic factors Bid,

Bak. Bad, Smac and p53 was observed in GRTH null mice while levels of anti-apoptotic proteins including Bcl2, Bcl-xL, phospho-Bad, and HSP10 were significantly reduced. Also, major reduction of PKA(c), Erk1/2 and pErk1/2, enzymes known to phosphorylate Bad (anti-apoptotic) was found in the GRTH null mice indicating the general effect of this helicase on the mitochondria directed apoptotic route (37). The lack of GRTH could reduce mitochondria integrity through the increases in membrane bound proapoptotic proteins that promote release of cytochrome C with significant effects on Caspase Signal Pathways. These included significant increases in active cleaved products of caspases 9 and 3 and PARP recognize to induce DNA fragmentation (**Table 1**). GRTH through its association with caspase 3 mRNA has a significant role in its stability through increased degradation, consequently marked increases in the half-life of the transcript was observed in the null mice. GRTH has effect on NFκB-mediated anti-apoptotic pathway. Increased levels of IκBα and reduction of its phosphorylation which cause sequestration of NFκB dimers in the cytoplasm was observed in the KO mice. This promotes association of IκBα/β with NFκB and prevents its nuclear translocation which is required for transcriptional activation of antiapoptotic genes. Moreover, GRTH regulates the TNFα/TNF-R1 mediated pathway and caspase 8 mediated events by regulating the levels of TRADD expression. GRTH associated with some apoptotic factors (Bad, Bac, Smac, p53) and anti-apoptotic factors (Bcl-2, Bcl-xl, HSp10). It also associates with caspases 3, 8, and 9, PARP, TRADD, and IκB and nuclear regulators of NFκB- p300 and HDAC1 (37). The prevalence of apoptotic or antiapoptotic pathways could result from association of corresponding transcripts with GRTH which in turn cause silencing/degradation or alter translation and transport events.

ROLE OF PHOSPHO-GRTH IN SPERMATOGENESIS

Our early studies revealed that a missense heterozygous mutation of R249 to H of GRTH found in 5.8% of Japanese patients with non-obstructive azoospermia and 1% of normal controls, when expressed in COS1 cells, causes loss of the 61 kDa cytoplasmic phospho-GRTH species with preservation of the nuclear 56 kDa non-phospho form (38). From this initial finding we could conclude that the mutation observed in these patients was not the

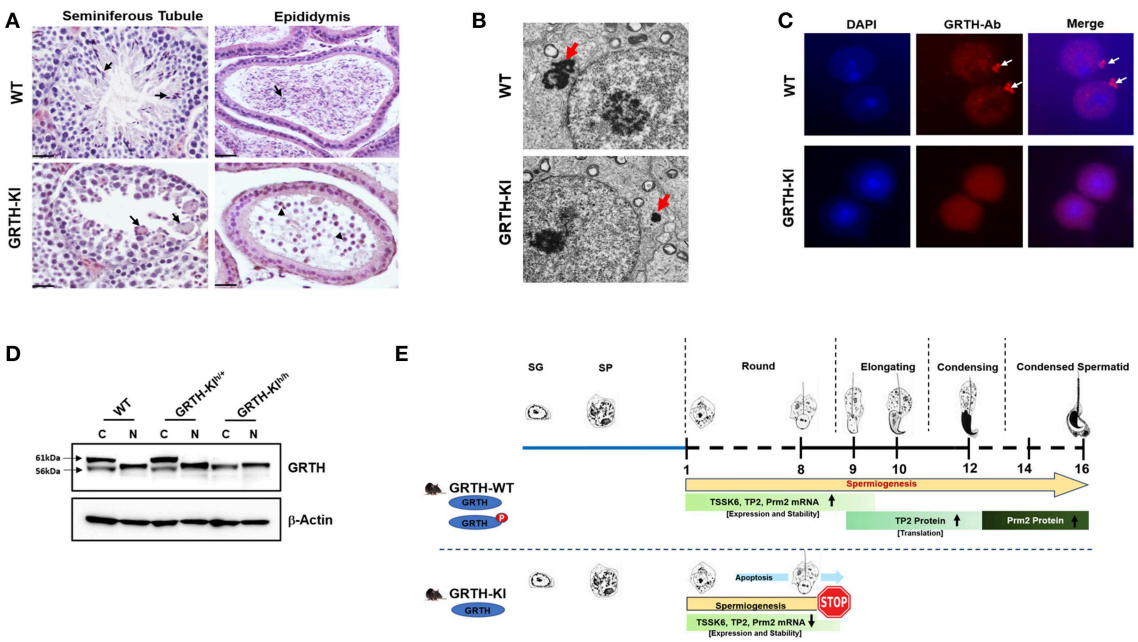

FIGURE 4 | GRTH-KI and essential role of phospho-GRTH in round spermatid during spermiogenesis. **(A)** H&E staining of WT and GRTH-KI mice testis showing seminiferous tubules and epididymis. In WT mice seminiferous tubules arrows indicate presence of elongated spermatids. GRTH-KI show degenerating multinucleated giant cells indicated by arrows. Epididymis of WT mice is filled with mature sperm (indicated in arrows) while in KI mice lack sperm but the lumens contain degenerating germ cells (indicated in arrowheads). **(B)** EM sections testis showing marked size reduction in chromatoid body (CB) in round spermatids (red arrows) of GRTH-KI mice compared to WT mice. **(C)** Immunofluorescence staining of GRTH protein (Red) in the nucleus and at cytoplasmic sites, in chromatoid bodies (CB) of round spermatids of WT mice (39). Arrows indicate CB. GRTH-KI mice lack GRTH signal in the CB. Nuclear staining using DAPI is shown in blue. **(D)** Western blot showing non-phospho GRTH expression in WT, heterozygous and homozygous KI mice while pGRTH expression was totally absent in homozygous KI mice compared to WT mice and heterozygous KI mice (39). **(E)** Schematic diagram showing progression of mice spermatogenesis from spermatogonia to round spermatids which undergo different steps (total 16) of development to give rise to a condensed sperm. In WT mice during the process of spermiogenesis in round spermatids we observed stable expression of Tssk6, Tp2, and Prm2 mRNAs and phospho-GRTH plays an important role in the stability of these germ cells specific mRNAs until ready for translation in later steps during elongation of spermatids. During spermatid elongation transition proteins are replaced by protamines1/2 and the chromatin becomes more condensed. While in GRTH-KI mice due to lack of phospho-GRTH the stability of above-mentioned germ cells specific mRNAs is hampered resulting in their reduced mRNA levels and degradation. In GRTH-KI mice Tp2 and Prm2 proteins are absent due to failure of round spermatids to elongate in step 8 of spermiogenesis (39).

cause of azoospermia, as judge by our observation in null mice where only homozygous null mice were infertile (38). However, the finding provided an avenue to elucidate the function of phospho-GRTH in spermatogenesis. Recently we generated a humanized GRTH knock-in (KI) mouse with the R242 to H mutation observed in the patients (39). Homozygous mice are sterile with marked reduction in the size of the testis which lack sperm (**Figure 4A**) with arrest at step 8 of round spermatids and complete loss of the cytosolic phospho-GRTH species (61 kDa) with preservation of the non-phospho form of GRTH (**Figure 4D**). In KI mice the androgen and gonadotropin levels were comparable to the Wild Type and the mating behavior is normal. In contrast, heterozygous mice are fertile. The non-phospho form is predominant in the nucleus and small quantities are also present in the cytoplasm of the KI mice. Thus, in KI mice the nuclear transport functions were preserved while the cytoplasmic functions including shuttling of messages, storage in the CBs and translational events which require phospho-GRTH are absent. Consequently, a marked reduction of the chromatoid body of round spermatids was revealed (**Figure 4B**). KI mice lack phospho-GRTH protein in CBs (**Figure 4C**). Germ cell apoptosis was observed in pachytene spermatocytes and round spermatids until the arrest point. In contrast to KO, KI mice revealed no changes in miRNA biosynthesis which indicated involvement of non-phospho rather than phospho-GRTH most likely as a transcriptional regulator of members of the microprocessor complex Drosha DCGR affecting primiRNAs formation (36). In KI mice we found loss of chromatin remodeling and related proteins including TP2, PRM2, and TSSK6 and also a significant decrease of their mRNAs and half-lives, indicating that their association with phospho-GRTH in the cytoplasm protect mRNAs from degradation (**Figure 4E**). Moreover, we demonstrated that mRNAs concerned in spermatogenesis bound phospho-GRTH and their association to actively translating polyribosomes. These and related transcripts were found down-regulated at polysomal sites in KO mice. Ingenuity analysis predicted association of pGRTH bound messages at polysomal sites of round spermatids with the ubiquitin-proteosome-heat shock protein network and the NFκB/TP53/TGFB1 signaling (40). In early studies we demonstrated that GRTH protein bind to the 3′ UTR region of mRNA (41). In very recent functional studies, we have shown that phospho-GRTH has an important role in the translation of Tp2 through binding of its 3′ untranslated regions (39).

THE GRTH PHOSPHO-SITE AND ITS CONNECTION WITH PKAα CATALYTIC -AS THE INITIAL STEP FOR DEVELOPMENT OF A NON-HORMONAL MALE CONTRACEPTIVE

The identification of the GRTH phospho-site at threonine (T239) (18) structurally adjacent to the mutant site (R 242H) found in patients (38) provided a frame for modeling of relevant amino-acids that form the GRTH pocket/PKAα catalytic interphase. Molecular modeling based on the RecA domain 1 of DDX19 (18, 42, 43) elucidated the relevant amino acids that formed the pocket, solvent accessibility and H-bonding, which upon disruption caused reduction or abolition of the phospho-GRTH form proven essential for completion of spermatogenesis. These include in addition of the core residues at T239 and R242 amino acids E165, K240, and D237. It is relevant to note that the deleterious effects on GRTH phosphorylaton caused by the mutations are not engendered from changes of PKAα-catalytic binding affinity but in the arrangement of the pocket for efficient catalytic activity of the kinase (18). Blocking the phosphorylation of GRTH at T239 through perturbation of the pocket should provide an effective selective and specific oral non-hormonal male contraceptive.

AUTHOR CONTRIBUTIONS

MD and RK conceived the concept of this review and wrote the manuscript.

FUNDING

This work was supported by the NIH Intramural Research Program through the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

REFERENCES

1. Tang PZ, Tsai-Morris CH, Dufau ML. A novel gonadotropin-regulated testicular RNA helicase. A new member of the dead-box family. *J Biol Chem.* (1999) 274:37932–40. doi: 10.1074/jbc.274.53.37932
2. Cigorraga SB, Dufau ML, Catt KJ., Regulation of luteinizing hormone receptors and steroidogenesis in gonadotropin-desensitized Leydig cells. *J Biol Chem.* (1978) 253:4297–304.
3. Dufau ML, Cigorraga SB, Baukal AJ, Bator JM, Sorrell SH, Neubauer JF, et al. Steroid biosynthetic lesions in gonadotropin-desensitized Leydig cells. *J Steroid Biochem.* (1979) 11:193–9. doi: 10.1016/0022-4731(79)90296-6
4. Nozu K, Dufau ML, Catt KJ. Estradiol receptor-mediated regulation of steroidogenesis in gonadotropin-desensitized Leydig cells. *J Biol Chem.* (1981) 256:1915–22.
5. Sheng Y, Tsai-Morris CH, Dufau ML. Cell-specific and hormone-regulated expression of gonadotropin-regulated testicular RNA helicase gene (GRTH/Ddx25) resulting from alternative utilization of translation initiation codons in the rat testis. *J Biol Chem.* (2003) 278:27796–803. doi: 10.1074/jbc.M302411200
6. Linder P. Dead-box proteins: a family affair–active and passive players in RNP-remodeling. *Nucleic Acids Res.* (2006) 34:4168–80. doi: 10.1093/nar/gkl468
7. Dufau ML, Tsai-Morris CH. Gonadotropin-regulated testicular helicase (GRTH/DDX25): an essential regulator of spermatogenesis. *Trends Endocrinol Metab.* (2007) 18:314–20. doi: 10.1016/j.tem.2007.09.001
8. Sheng Y, Tsai-Morris CH, Gutti R, Maeda Y, Dufau ML. Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene-specific mRNA export and protein translation during spermatogenesis. *J Biol Chem.* (2006) 281:35048–56. doi: 10.1074/jbc.M605086200
9. Gee SL, Conboy JG. Mouse erythroid cells express multiple putative RNA helicase genes exhibiting high sequence conservation from yeast

to mammals. *Gene.* (1994) 140:171–7. doi: 10.1016/0378-1119(94)90541-X
10. Tseng SS, Weaver PL, Liu Y, Hitomi M, Tartakoff AM, Chang TH. Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export. *EMBO J.* (1998) 17:2651–62. doi: 10.1093/emboj/17.9.2651
11. Lin DH, Correia AR, Cai SW, Huber FM, Jette CA, Hoelz A. Structural and functional analysis of mRNA export regulation by the nuclear pore complex. *Nat Commun.* (2018) 9:2319. doi: 10.1038/s41467-018-04459-3
12. Williams-Hill DM, Duncan RF, Nielsen PJ, Tahara SM. Differential expression of the murine eukaryotic translation initiation factor isogenes eIF4A(I) and eIF4A(II) is dependent upon cellular growth status. *Arch Biochem Biophys.* (1997) 338:111–20. doi: 10.1006/abbi.1996.9804
13. Askjaer P, Bachi A, Wilm M, Bischoff FR, Weeks DL, Ogniewski V, et al. RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. *Mol Cell Biol.* (1999) 19:6276–85. doi: 10.1128/MCB.19.9.6276
14. Chuang RY, Weaver PL, Liu Z, Chang TH. Requirement of the DEAD-Box protein ded1p for messenger RNA translation. *Science.* (1997) 275:1468–71. doi: 10.1126/science.275.5305.1468
15. Noce T, Okamoto-Ito S, Tsunekawa N. Vasa homolog genes in mammalian germ cell development. *Cell Struct Funct.* (2001) 26:131–6. doi: 10.1247/csf.26.131
16. Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, et al. The mouse homolog of Drosophila Vasa is required for the development of male germ cells. *Genes Dev.* (2000) 14:841–53.
17. Kavarthapu R, Tsai-Morris CH, Fukushima M, Pickel J, Dufau ML. A 5'-flanking region of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) gene directs its cell-specific androgen-regulated gene expression in testicular germ cells. *Endocrinology.* (2013) 154:2200–7. doi: 10.1210/en.2012-2230
18. Raju M, Hassan SA, Kavarthapu R, Anbazhagan R, Dufau ML. Characterization of the phosphorylation site of GRTH/DDX25 and protein kinase a binding interface provides structural basis for the design of a non-hormonal male contraceptive. *Sci Rep.* (2019) 9:6705. doi: 10.1038/s41598-019-42857-9
19. Karabulut NP, Frishman D. Sequence- and structure-based analysis of tissue-specific phosphorylation sites. *PLoS ONE.* (2016) 11:e0157896. doi: 10.1371/journal.pone.0157896
20. Tsai-Morris CH, Lei S, Jiang Q, Sheng Y, Dufau ML. Genomic organization and transcriptional analysis of gonadotropin-regulated testicular RNA helicase–GRTH/DDX25 gene. *Gene.* (2004) 331:83–94. doi: 10.1016/j.gene.2004.02.004
21. Villar J, Tsai-Morris CH, Dai L, Dufau ML. Androgen-induced activation of gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) transcription: essential role of a nonclassical androgen response element half-site. *Mol Cell Biol.* (2012) 32:1566–80. doi: 10.1128/MCB.06002-11
22. Kavarthapu R, Dufau ML. Germ cell nuclear factor (GCNF/RTR) regulates transcription of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) in testicular germ cells–the androgen connection. *Mol Endocrinol.* (2015) 29:1792–804. doi: 10.1210/me.2015-1198
23. Tsai-Morris CH, Sheng Y, Lee E, Lei KJ, Dufau ML. Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is essential for spermatid development and completion of spermatogenesis. *Proc Natl Acad Sci USA.* (2004) 101:6373–8. doi: 10.1073/pnas.0401855101
24. Fukushima M, Villar J, Tsai-Morris CH, Dufau ML. Gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), a negative regulator of luteinizing/chorionic gonadotropin hormone-induced steroidogenesis in Leydig cells: central role of steroidogenic acute regulatory protein (StAR). *J Biol Chem.* (2011) 286:29932–40. doi: 10.1074/jbc.M111.236083
25. Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. *Nat Rev Mol Cell Biol.* (2007) 8:85–90. doi: 10.1038/nrm2081
26. Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, et al. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. *Proc Natl Acad Sci USA.* (1994) 91:12258–62. doi: 10.1073/pnas.91.25.12258
27. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Takamatsu K, Chuma S, Kojima-Kita K, et al. MVH in piRNA processing and gene silencing of retrotransposons. *Genes Dev.* (2010) 24:887–92. doi: 10.1101/gad.1902110
28. Sato H, Tsai-Morris CH, Dufau ML. Relevance of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) in the structural integrity of the chromatoid body during spermatogenesis. *Biochim Biophys Acta.* (2010) 1803:534–43. doi: 10.1016/j.bbamcr.2010.02.004
29. Lehtiniemi T, Kotaja N. Germ granule-mediated RNA regulation in male germ cells. *Reproduction.* (2018) 155:R77–91. doi: 10.1530/REP-17-0356
30. Fawcett DW, Leak LV, Heidger PM Jr. Electron microscopic observations on the structural components of the blood-testis barrier. *J Reprod Fertil Suppl.* (1970) 10:105–22.
31. Russell L, Frank B. Ultrastructural characterization of nuage in spermatocytes of the rat testis. *Anat Rec.* (1978) 190:79–97. doi: 10.1002/ar.1091900108
32. Gou LT, Dai P, Yang JH, Xue Y, Hu YP, Zhou Y, et al. Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis. *Cell Res.* (2014) 24:680–700. doi: 10.1038/cr.2014.41
33. Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol.* (2007) 27:3970–81. doi: 10.1128/MCB.00128-07
34. Decker CJ, Parker R. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harb Perspect Biol.* (2012) 4:a012286. doi: 10.1101/cshperspect.a012286
35. Ma W, Mayr C. A membraneless organelle associated with the endoplasmic reticulum enables 3'UTR-mediated protein-protein interactions. *Cell.* (2018) 175:1492–506.e19. doi: 10.1016/j.cell.2018.10.007
36. Dai L, Tsai-Morris CH, Sato H, Villar J, Kang JH, Zhang J, et al. Testis-specific miRNA-469 up-regulated in gonadotropin-regulated testicular RNA helicase (GRTH/DDX25)-null mice silences transition protein 2 and protamine 2 messages at sites within coding region: implications of its role in germ cell development. *J Biol Chem.* (2011) 286:44306–18. doi: 10.1074/jbc.M111.282756
37. Gutti RK, Tsai-Morris CH, Dufau ML. Gonadotropin-regulated testicular helicase (DDX25), an essential regulator of spermatogenesis, prevents testicular germ cell apoptosis. *J Biol Chem.* (2008) 283:17055–64. doi: 10.1074/jbc.M708449200
38. Tsai-Morris CH, Koh E, Sheng Y, Maeda Y, Gutti R, Namiki, M. et al. Polymorphism of the GRTH/DDX25 gene in normal and infertile Japanese men: a missense mutation associated with loss of GRTH phosphorylation. *Mol Hum Reprod.* (2007) 13:887–92. doi: 10.1093/molehr/gam065
39. Kavarthapu R, Anbazhagan R, Raju M, Morris CT, Pickel J, Dufau ML. Targeted knock-in mice with a human mutation in GRTH/DDX25 reveals the essential role of phosphorylated GRTH in spermatid development during spermatogenesis. *Hum Mol Genet.* (2019) 28:ddz079. doi: 10.1093/hmg/ddz079
40. Tsai-Morris CH, Sato H, Gutti R, Dufau ML. Role of gonadotropin regulated testicular RNA helicase (GRTH/Ddx25) on polysomal associated mRNAs in mouse testis. *PLoS ONE.* (2012) 7:e32470. doi: 10.1371/journal.pone.0032470
41. Yang R, Tsai-Morris CH, Kang JH, Dufau ML. Elucidation of RNA binding regions of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) to transcripts of a chromatin remodeling protein essential for spermatogenesis. *Horm Mol Biol Clin Investig.* (2015) 22:119–30. doi: 10.1515/hmbci-2015-0004
42. von Moeller H, Basquin C, Conti E. The mRNA export protein DBP5 binds RNA and the cytoplasmic nucleoporin NUP214 in a mutually exclusive manner. *Nat Struct Mol Biol.* (2009) 16:247–54. doi: 10.1038/nsmb.1561
43. Collins R, Karlberg T, Lehtiö L, Schütz P, van den Berg S, Dahlgren LG, et al. The DEXD/H-box RNA helicase DDX19 is regulated by an {alpha}-helical switch. *J Bio Chem.* (2009) 284:10296–300. doi: 10.1074/jbc.C900018200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

CORRECTION
published: 18 October 2019
doi: 10.3389/fendo.2019.00710

Corrigendum: Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 September 2019*
Accepted: *02 October 2019*
Published: *18 October 2019*

Citation:
Dufau ML and Kavarthapu R (2019) Corrigendum: Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive. Front. Endocrinol. 10:710. doi: 10.3389/fendo.2019.00710

Keywords: GRTH/DDX25, spermatogenesis, round spermatids, androgen, hormonal regulation

A Corrigendum on

Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive

by Dufau, M. L., and Kavarthapu, R. (2019). Front. Endocrinol. 10:576. doi: 10.3389/fendo.2019.00576

In the original article, there was an error in the ***Title***, first instance of the word "Regulation" should have been "Regulated."

A correction has been made to ***Title***:

"Gonadotropin Regulated Testicular RNA Helicase, Two Decades of Studies on Its Structure Function and Regulation From Its Discovery Opens a Window for Development of a Non-hormonal Oral Male Contraceptive"

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Endocrinology

MINI REVIEW
published: 04 September 2019
doi: 10.3389/fendo.2019.00609

Targeting Endothelin-1 Receptor/β-Arrestin-1 Axis in Ovarian Cancer: From Basic Research to a Therapeutic Approach

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Recent studies imply a key role of endothelin-1 receptor (ET-1R), belonging to the largest family of G protein-coupled receptors (GPCR), in the regulation of a plethora of processes involved in tumorigenesis and metastatic progression. β-arrestin-1 (β-arr1) system has been recognized as a critical hub controlling GPCR signaling network, directing the GPCR's biological outcomes. In ovarian cancer, ET-1R/β-arr1 axis enables cancer cells to engage several integrated signaling, and represents an actionable target for developing novel therapeutic approaches. Preclinical research studies demonstrate that ET-1R blockade by the approved dual ET_AR/ET_BR antagonist macitentan counteracts β-arr1-mediated signaling network, and hampers the dialogue among cancer cells and the tumor microenvironment, interfering with metastatic progression and drug response. In light of major developments in the ET-1R signaling paradigm, this review article discusses the emerging evidence of the dual ET-1R antagonist treatment in cancer, and outlines our challenge in preclinical studies warranting the repurposing of ET-1R antagonists for the design of more effective clinical trials based on combinatorial therapies to overcome, or prevent, the onset of drug resistance.

Keywords: endothelin-1, endothelin-1 receptors, ovarian cancer, β-arrestin-1, G-protein coupled receptors

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Edited by:
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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *14 June 2019*
Accepted: *21 August 2019*
Published: *04 September 2019*

Citation:
Tocci P, Rosanò L and Bagnato A (2019) Targeting Endothelin-1 Receptor/β-Arrestin-1 Axis in Ovarian Cancer: From Basic Research to a Therapeutic Approach. Front. Endocrinol. 10:609. doi: 10.3389/fendo.2019.00609

INTRODUCTION

G protein-coupled receptors (GPCR) are major therapeutic targets because their signaling, influencing many cellular biochemical activities, phenotype plasticity, and gene expression, touches numerous aspects of human endocrinology, physiology, and pathophysiology, including tumorigenesis (1, 2). Among these, the endothelin-1 (ET-1) receptors (ET-1R), ET_A receptor (ET_AR), and ET_B receptor (ET_BR), exert critical functions in many tumor settings and their aberrant expression has been observed in several malignances including ovarian cancer (OC) (3). Surgery and platinum-based chemotherapy represent the standard approach for OC patients. Clinical evidences indicate that even after initial response, the majority of OC patients relapse and acquire chemotherapy resistance (4–6). The limited clinical response observed in OC patients may be related to the integration network of different pathways, including ET-1 signaling. ET-1R activation occurs through the agonist-dependent binding of three 21-amino acid isoforms, ET-1, ET-2, and ET-3 (7–9), and their eclectic effects are due to the different grades of affinity of ET_AR and ET_BR for the three ligands (7–12). Indeed, ET_AR exhibit higher affinity for ET-1 and ET-2, while ET_BR binds the three ETs isoforms with equal affinity (8).

ET-1R activation mechanism have long been reported as a process consisting of the ETs bound to the receptor and G protein coupling, which triggers a signaling cascade including an increase in intracellular Ca^2, activation of Rho-kinase, or adenylate cyclase/cyclic adenosine monophosphate pathway, and the transactivation of the epidermal growth factor receptor (EGFR) (3, 13, 14).

In addition to activating the G-protein pathway, ET-1R activate the β-arrestins (β-arr) pathway, known as β-arrestin-1 (β-arr1) and β-arrestin-2 (β-arr2), which guiding ET-1R internalization by the engagement of clathrin-coated pits, directs ET-1R recycling or lysosomal degradation, precluding G-protein coupling even in presence of a persistent ET-1 stimulus (15–18). While both ET-1R are internalized by β-arr and clathrin-dependent mechanisms, ET_AR are recycled to the cell membrane while ET_BR are lysosomal degraded (19). Interestingly, increasing evidence points out the existence of a signaling concept by which ET-1R reinforces and prolongs its signaling pattern through β-arr1, which favoring multi-protein complex formation, defines new signaling outputs parallel to the traditional G-protein-dependent one (3, 20). In particular, β-arr1 converts protein-protein interaction into a connected signaling network counting several oncogenic pathways, in both the cytoplasm and the nucleus (3, 20).

Downstream of ET-1R, the recognition of this β-arr1 role may be particularly relevant for the design of novel therapeutic combinations able to efficiently hamper also the ET-1R/β-arr1-dependent branch, besides the G-protein-mediated routes (3). In this scenario, this review aims to portray the multiple facet of ET-1-dependent signaling, describing the impact of β-arr1 activity on the integration between ET-1R signaling with other regulatory pathways which may influence both the tumor and the surrounded TME. The availability of appropriate preclinical models will allow more reliable studies in physio-pathological complex context to validate more effective therapeutic approaches able to antagonize the ET-1R/β-arr1 signaling network in OC and other malignancies.

THE ROLE OF ET-1R/β-ARR1-MEDIATED FUNCTIONS IN OVARIAN CANCER

Integrated Network of ET-1R/β-arr1-Mediated Signaling and Other Pathways

The integration of diverse signaling pathways operating concurrently within tumor cells may represent a mechanism to escape drug response. In this view, the identification of interconnected pathways, as critical vulnerabilities that allow OC progression and chemotherapy resistance, represents an essential tool to design promising therapeutic combination against OC. An increasing body of evidence has well-documented the close connection between ET-1/ET-1R axis and other signaling pathways in cancer. Indeed, β-arr1 may influence ET-1R signaling, physically complexing with multiple signal transducers, which exhibit different functions and localization, including cytoplasmic and nuclear proteins (3, 21–24), as well as cytoskeletal components (25–27), therefore promoting the integration of ET-1R signaling with different intracellular pathways. By acting as hub able to engage a pool of oncogenic intermediates, β-arr1 controls in a fine-tuning manner several aspects of ET-1-induced functions related to OC progression, including cell growth, cell survival, invasion, and chemoresistance (3). An example of how β-arr1, mediating the cross-talk of GPCR with other receptors, fosters ET-1R signaling network sophistication is represented by the crosstalk between ET-1R and the receptor tyrosine kinase (RTK) family members, as EGFR or the vascular endothelial growth factor receptor-3 (VEGFR-3) (28), through the recruitment of SRC, that promotes the transactivation of RTK in OC cells (3, 28).

Recent studies reported that β-arr1 signaling, converging on cytoskeleton remodeling-related signaling routes, generates dynamic morphological changes which produce the force required for changes in cell shape leading to cell invasion (29). In this regard, it has been demonstrated that downstream of ET-1R, β-arr1 activates a signaling cascade by interacting with PDZ-RhoGEF which, in turn, induces RhoC GTPase and cofilin pathway, and the formation of actin-rich invasive protrusions named invadopodia (25). β-arr1-associated molecular complexes during invadopodia maturation require the interaction with ENA/VASP family members, such as hMENA that regulates the cytoskeleton dynamic behavior of different cell types (26). The activation of ET-1R promotes the formation of a ternary complex consisting of β-arr1/hMENA/PDZ-RhoGEF which induces RhoC signaling, mediating extracellular matrix (ECM) degradation (26). More recently, it has been disclosed a new mechanistic association among ET-1R/β-arr1 axis and the integrin-related protein IQ-domain GTPase-activating protein 1 (IQGAP1) that participates to cytoskeleton remodeling, and invadopodia-dependent ECM degradation (27). In particular, β-arr1/IQGAP1 complex induces Rac1 inhibition and a concomitant RhoA and RhoC activation, indicating that ET-1R-driven β-arr1 interactions regulate the specific inputs for invadopodia formation and activation (27). This aspect further updates the landscape of the ET-1R/β-arr1-integrated pathways and may magnify the understanding of the specific β-arr1 contribution to such network complexity.

A noteworthy β-arr1-mediated cross-talk is that between ET-1R axis and β-catenin signaling. ET-1R activation induces β-arr1 recruitment at the cell membrane, where β-arr1 may trigger β-catenin stabilization through two modalities: the first one promoting the EGFR transactivation via SCR, β-catenin tyrosine phosphorylation and in turn its activation; the second one by inhibiting the β-catenin destruction complex, constituted by glycogen synthase kinase-3β (GSK-3β), axis inhibition 1 (AXIN1), adenomatous polyposis coli (APC), and β-transducin repeat containing protein (β-TrCP), therefore promoting β-catenin activation (30). Of note, the β-arr1-dependent ET-1R/β-catenin signaling integration is not limited to the cytoplasm. Indeed, this signaling interplay culminates into the nucleus where β-arr1/β-catenin physical interaction leads to β-catenin nuclear translocation, p300 recruitment on β-catenin target genes promoters, favoring chromatin acetylation and transcriptional

activity of β-catenin/T-cell factor 4 (TCF4) that promotes gene expression, including *EDN1* (ET-1 gene), fueling an autocrine loop that sustain a persistent β-catenin activation, that fosters chemoresistance and metastatic behavior (21, 22).

More recently, it has been reported that the nuclear binding of β-arr1 to the hypoxia-inducible factor-1α (HIF-1α) creates a new signaling channel that connects ET-1R pathway to HIF-1α activity. In OC cells, β-arr1, as a nuclear co-factor, directs the recruitment of HIF-1α, as well as of p300, on hypoxia responsive elements contained within HIF-1α target gene promoters, promoting the transcription of pro-angiogenic genes, such as *EDN1* and *VEGF* (23). Consistent with these results, a ChIP-Seq analysis in prostate cancer cells exposed to pseudo-hypoxic conditions highlights the partial overlay of binding sites of β-arr1 and p300 within promoters and intronic regions. The identification of non-overlapping sites suggests that β-arr1 may regulate gene transcription also autonomously from p300 (31). In addition, the analysis of both β-arr1 and HIF-1α transcriptomes in breast cancer shows the overlap of the two gene profiles, including known HIF-1α targets such as those required in neovascularization, and aerobic glycolysis (32).

Similarly, it has been reported that β-arr1 allows the interfacing of ET-1R axis with the nuclear factor κB (NF-κB) signaling in OC cells. In particular, ET-1R activation, in a β-arr1-dependent manner, induces the phosphorylation of p65 leading to its activation and in parallel IκB-α phosphorylation, inducing its degradation. These two steps are critical for the β-arr1-dependent p65 nuclear accumulation and transcriptional activity. The existence of the cross-talk between ET-1R/β-arr1 and NF-κB provide a further attempt of how ET-1R/β-arr1 axis may affect chromatin remodeling and gene transcription regulation (24).

Regarding the ability of β-arr1 to interconnect oncogenic signaling pathways, a recent study discloses the interplay between ET-1R/β-arr1 axis and the Hippo transducers YAP and TAZ in patient-derived high-grade serous ovarian cancer (HG-SOC) cells and in breast cancer cell lines carrying *TP53* mutations (mutp53) (33), indicating a therapeutic option for the treatment of mutp53 cancers and opening new prospects on the regulation of YAP/TAZ biology (34, 35). Mechanistically, β-arr1 engages a physical interaction with the two related transcription co-activators YAP and TAZ into the cytoplasm where mediates its de-phosphorylation, leading to YAP/TAZ cytoplasmic-nuclear shuttling and activation in a G-protein independent manner. In parallel, β-arr1 interacting with a RhoGEF family member, Trio, integrates an additional signaling route that includes RhoA GTPase, and actin cytoskeleton activity, further favoring YAP/TAZ nuclear accumulation. As co-transcriptional factor, YAP binds TEAD as critical oncogenic transcription factor (36). In the nucleus, β-arr1 enrolling mutp53 on YAP/TEAD target gene promoters, becomes part of a ternary complex consisting of β-arr1/YAP/mutp53 that induces the aberrant expression of TEAD target genes. Remarkably, this transcriptionally active complex mediates also *EDN1* transcription, thus magnifying a positive feed-back loop that sustains a persistent YAP activation, which induces cell proliferation, survival and invasion. Moreover, in breast cancer cells β-arr1 may anchor also NFY transcriptional factor together with YAP and mutp53 (33, 37), inducing the transcription of the proliferative genes, further expanding the repertoire of β-arr1 nuclear partners and proving that β-arr1/mutp53 cooperation may support the transcription of YAP-associated signature. Clinically relevant, the expression of ET-1, ET_AR, and YAP were simultaneously up-regulated in HG-SOC tissues compared to normal ovarian tissues. Additionally, HG-SOC patients harboring *TP53* mutations, with a combined high expression levels of ET-1R/β-arr1/YAP have a worse prognosis compared to patients who lack this network-based signature, emphasizing the poor outcomes generated by the integration between ET-1R/β-arr1 and YAP pathways and contribute to identify a predictive gene signature for recurrent HG-SOC (33).

These findings highlight the multimodality by which β-arr1, acting not only as a cytoplasmic signal transducer but, more significant, as a dynamic nuclear linker that guides the positioning of transcriptional factor and co-factors, regulates epigenetic control and, in turn, defines a highly characteristic transcriptional profile, creating an additional layer of ET-1R signaling regulation in oncogenic transcriptional activity (**Table 1**).

ET-1R/β-arr1-Mediated Cross-Talk With Other Signaling in the Tumor-Microenvironment

The interfacing of transformed cells with the surrounded tumor-associated elements from the TME, which include cancer-associated fibroblasts (CAF), endothelial cells (EC), and immune cells, as lymphocytes and tumor-associated macrophages (TAM), as well as the interaction between tumor cells and the ECM, impacts on cancer growth, progression and clinical outcome. A deep knowledge of how the dialogue among tumor cells, TME elements, and ECM may shape clinical response is an unmet medical need in oncology. Regarding this aspect, emerging data indicate that ET-1 signaling has a significant influence on several pathways and cellular processes involving both the tumor and the TME, thus emerging as a regulator of the signaling interchange between tumor and stromal compartment. In this perspective, it has been demonstrated that ET-1 is a regulator of tumor stroma. Indeed, tumor cells produce and secrete ET-1 which may facilitate tumor stroma remodeling (38, 39). In particular in many tumor type, including OC, it has been reported that ET-1 activating the ET-1R, both expressed by fibroblasts isolated by normal tissues near to cancer tissues, promote their growth, migration and contraction, as well as the production of ECM modifying factors (40), suggesting that such cross-talk may take place through the paracrine release of ET-1 that, fostering the formation of a prone tumor stroma, participates to create a niche that support tumor maintenance.

In addition, ET-1 seems to regulate also the immune environment at different levels, acting on different subclasses of immune effectors. Indeed, ET-1 appears to modulate dendritic cells (DC) behavior and activity (41). Moreover, a transcriptional profile conducted in OC shows that the over-expression of ET_BR is negatively associated to the recruitment of tumor-infiltrating lymphocytes (TIL), which depends on the ET_BR-dependent reduction of endothelial intercellular adhesion molecule 1

TABLE 1 | Endothelin-1-induced β-arrestin-1 cooperation with transcription factors in cancer cells.

Transcription factor	Biological role	Transcriptional effect	References
β-catenin/TCF4	Induction of migration, invasion, epithelial-to-mesenchymal transition, chemoresistance, vascularization, intravasation, and metastatic progression in ovarian cancer cells	ACTIVATION	(21, 22)
HIF-1α	Promotion of pro-tumorigenic behavior in ovarian, prostate and breast cancer cells and induction of pro-angiogenic effects in endothelial cells	ACTIVATION	(23, 31, 32)
NFκB	Induction of cell survival in ovarian cancer cells	ACTIVATION	(24)
YAP/TEAD	Induction of cell proliferation, survival, and invasion in patient-derived high-grade serous ovarian cancer cells and breast cancer cells carrying *TP53* mutations	ACTIVATION	(33)
Mutant p53	Promotion of cell proliferation, survival, and invasion in patient-derived high-grade serous ovarian cancer cells and breast cancer cells carrying *TP53* mutations	ACTIVATION	(33)
NFY	Induction of cell proliferation in breast cancer cells carrying *TP53* mutation	ACTIVATION	(33)

(ICAM1) expression. In line with this, ET_BR interfering increases the adhesion of T cells to the endothelium, favoring the homing of T cells to the tumor (42–44). These observations indicate that ET-1R potentially may control the ongoing immune response in the TME.

As above reported, β-arr1 acts as an initial activator of the cross-talk between ET-1R and RTK, including the VEGFR-3 (3). In addition, both ET_BR and VEGFR-3, as well as their associated ligands, ET-1, VEGF-A, VEGF-C and -D, act as environmental regulators affecting the blood and lymphatic endothelial cells (LEC) behavior (45, 46). In detail, it has been proved that ET-1, mediating ET_BR activation and cooperating with hypoxia, induces the release of pro-angiogenic and lymphangiogenic factors, as VEGF-A -C -D expression and, in turn promotes EC and LEC growth and invasion inducing neo-angiogenesis and lymphangiogenesis (46). More relevant, the dual capacity of ET-1/VEGF inter-relation of simultaneously influencing the tumor and the TME emerges also in their ability to control the mutual regulation between tumor cells, EC, LEC, and hypoxia, directing at the same time tumor aggressiveness and angiogenic activities. In the tumor, hypoxia induces ET-1 expression, along with VEGF-A and -C release, through HIF-1α and HIF-2α (3, 45, 46). The autocrine/paracrine interchange of these angiogenic factors, promotes tumor progression and morphological changes in EC and LEC, revealing that such signaling interplay sustains tumor development in a permissive TME. Considering that tumor cells, EC and LEC express ET_BR and VEGFR-3 as well as their cognate ligands, and taking into account that β-arr1 may coordinate the cooperation between ET_BR- and VEGFR-3-related pathways (46), it's tempting to speculate that β-arr1 may actively take part also to the ET-1/VEGF-induced bidirectional communications between LEC, EC, and tumor cells. The above hypothesis may be further supported by a novel mechanism by which β-arr1, directly regulating VEGFR-3 signaling and expression in human microvascular EC from lung, favors the development of pulmonary arterial hypertension (PAH) (47). Indeed, it has been reported that β-arr1 interferes with VEGFR-3 internalization and degradation, promoting its signaling. In line with this, β-arr1 knockout mice develop acute PAH that is related to the loss of VEGFR-3 signaling (47).

For all the above mentioned findings, we can consider ET-1R/β-arr1 system as a complex signaling machinery for the tumor/endothelial/immune cells input interchange in the tumor milieu, sustaining angiogenesis, lymphangiogenesis, and immune system control, thus providing an advantage to cancer cells to growth and metastasize.

INTERFERING WITH ET-1/β-ARR1 SIGNALING NETWORK FOR NEW THERAPEUTIC OPTIONS IN OVARIAN CANCER

In this review, an array of pathophysiological roles for the β-arr1-mediated pathways upon ET-1R activation in ovarian cancer is described (**Figure 1**). Thus, ET_AR/β-arr1 axis transmits signals to the nucleus, fostering early and late steps essential to metastatic progression and drug resistance. Therefore, besides the complexity of G-protein-mediated signaling (48), the disruption of the ET_AR/β-arr1 interaction can impair several hallmarks of cancer, representing a possible avenue for therapeutic intervention. The biased agonist that can transduce ET-1 signaling either through G-protein or β-arr1 leads to the new paradigm of signal-biased antagonists (49–51). The therapeutic strategy to block ET-1 activities has evolved in the clinic, mainly for PAH, by using orally active small molecule antagonists, targeting selectively ET_AR or both ET_AR and ET_BR. Besides the small molecules, other chemical compounds have been developed to target ET-1R, including monoclonal antibody antagonists and selective peptide agonists and antagonists (51). Different human cancers featured the benefit of targeting both ET_AR and ET_BR, in which ET-1R blockade with the dual ET_AR/ET_BR small molecule antagonists represents a suitable therapeutic option for ET-1R-expressing tumors. Indeed, the dual ET-1R antagonist macitentan concurrently attack tumor cells, which mainly express ET_AR, and TME elements, that express ET_BR, increasing anti-tumor immune and anti-angiogenic effects. In OC, silencing of β-arr1 or macitentan treatment, interfering with the interconnected pathways, inhibits tumor growth, angiogenesis (23), invasion, intravasation, and metastatic behavior (25–27), and sensitizes to platinum-based therapies (3, 22, 23). Additionally, macitentan is able to sensitize tumor cells to different cytotoxic and targeted agents in various preclinical tumor models, including colorectal

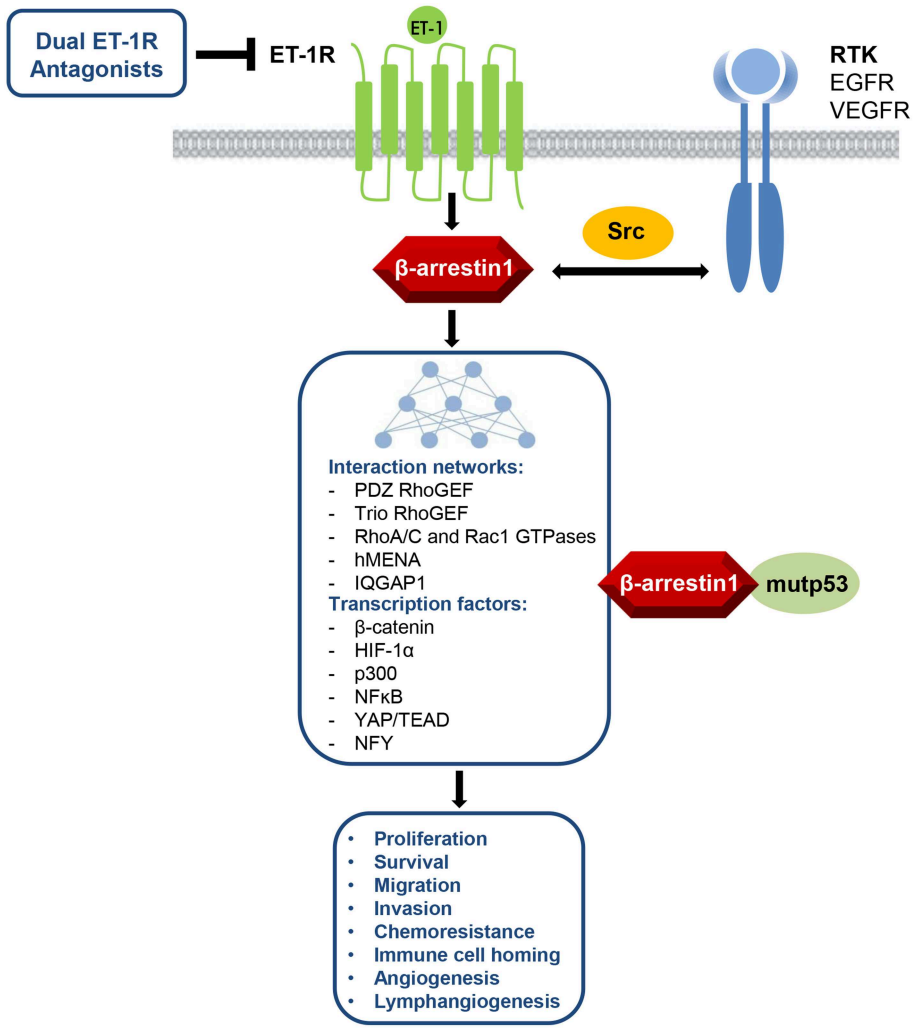

FIGURE 1 | ET-1R/β-arr1 acts as signaling hub regulating several cancer hallmarks via its interaction networks between GPCR and RTK pathways with different cytosolic, cytoskeletal, or nuclear signaling nodes, including also the control of mutant *TP53* (mutp53) oncogenic activity. This knowledge makes ET-1R/β-arr1 an attractive target for therapeutic intervention by using dual ET-1R antagonists.

cancer, glioblastoma, multiple myeloma, breast, and lung brain metastasis (3, 52–56). Future studies will guide to discover new biased antagonist with improved clinical profile. In this regard, the expected results of a phase III study (57, 58), that will explore the use of the long-acting (half-life of $\geq$12 h) aprocitentan, the active metabolite of the macitentan, in treatment-resistant hypertension, might indicate that a new licensed agent can be drug repurposed in oncology.

Since β-arr-1-mediated network might take place concomitantly in EC, LEC, fibroblasts, or in immune cells contributing to promote tumor progression in a suitable metastatic niche, future studies should also include reliable models that can recapitulate the complex TME. Therefore, the development of a platform of patient derived-preclinical models, including primary cell cultures, co-cultures with stromal elements, 3D organoids/tumoroids and patient-derived xenografts (PDX) is needed. These preclinical models, in particular those able to mimic the complexity of the TME landscape, as tumoroids (59), could be pivotal for modeling primary human tumor *ex vivo* and for drug screening. Considering the cancer vulnerability of ET-1R system and the mechanisms by which ET-1R antagonists synergize with other compounds (22, 52–56), further exploitation of the potential therapeutic paradigm of ET-1R blockade in combinatorial approaches in cancer now merits clinical consideration.

CONCLUSIONS

The ET-1R/β-arr1 axis integrates signaling pathways related to several hallmarks of cancer, entailing tumor cells and TME elements, thus contributing to tumor growth, metastasis formation and drug response. Therefore, further insights on the role of ET-1R/β-arr1 axis may favor the development of novel

effective therapies. In this context, the analysis of ET-1R/β-arr1 expression and β-arr1 interactome in OC and in different cancer types might be explored. The ability of β-arr1 to orchestrate the signaling network activated by ET-1/ET_AR pathway has been revealed especially in the context of drug resistance that requires the inception of escape pathways through the interplay with RTK and other oncogenic nodes. In this context, the blockade of ET-1R/β-arr1 axis, impairing different signaling cascades overcomes compensatory mechanism of chemotherapy escape. Considering the complexity of ET-1R/β-arr1-driven signaling networks, further studies should elucidate whether combinatorial targeted approaches using dual ET-1R antagonists with other chemotherapeutic or targeted agents, would enable overcoming drug resistance. These studies should also advance our understanding of how ET-1R/β-arr1 related functions are integrated in specific tumor and TME cell types cooperating with oncogenic drivers or enabling signaling networks to potentiate tumor progression, metastasis, and drug response.

Additionally, the discovery of new biomarkers predictive of drug response will allow patient selection and will help in defining more effective combined treatments. In future multidisciplinary studies, the integration of genomic, transcriptomic, proteomic, metabolomic data, taking into consideration β-arr1 as crucial member of GPCR-mediated pathways, would provide a more detailed understanding of downstream signaling in cancer, which would facilitate the design of new effective combination therapies.

AUTHOR CONTRIBUTIONS

PT, LR, and AB conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC) to AB (AIRC IG18382) and LR (AIRC IG21372).

ACKNOWLEDGMENTS

We gratefully acknowledge all members of the laboratory for their constant support and enthusiasm, and Maria Vincenza Sarcone for secretarial assistance.

REFERENCES

1. Chan HCS, Li Y, Dahoun T, Vogel H, Yuan S. New binding sites, new opportunities for GPCR drug discovery. *Trends Biochem Sci.* (2019) 44:312–30. doi: 10.1016/j.tibs.2018.11.011
2. Insel PA, Sriram K, Wiley SZ, Wilderman A, Katakia T, McCann T, et al. GPCRomics: GPCR expression in cancer cells and tumors identifies new, potential biomarkers and therapeutic targets. *Front Pharmacol.* (2018) 9:431. doi: 10.3389/fphar.2018.00431
3. Rosanò L, Spinella F, Bagnato A. Endothelin 1 in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer.* (2013) 13:637–51. doi: 10.1038/nrc3546
4. Torre LA, Trabert B, De Santis CE, Miller KD, Samimi G, Runowicz CD, et al. Ovarian cancer statistics. *CA Cancer J Clin.* (2018) 68:284–96. doi: 10.3322/caac.21456
5. Lheureux S, Gourley C, Vergote I, Oza AM. Epithelial ovarian cancer. *Lancet.* (2019) 393:1240–53. doi: 10.1016/S0140-6736(18)32552-2
6. Bast RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer.* (2009) 9:415–28. doi: 10.1038/nrc2644
7. Thompson MD, Cole DE, Capra V, Siminovitch KA, Rovati GE, Burnham WM, et al. Pharmacogenetics of the G protein-coupled receptors. *Methods Mol Biol.* (2014) 1175:189–242. doi: 10.1007/978-1-4939-0956-8_9
8. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* (1988) 332:411–5. doi: 10.1038/332411a0
9. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, et al. Endothelin. *Pharmacol Rev.* (2016) 68:357–418. doi: 10.1124/pr.115.011833
10. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA.* (1989) 86:2863–7. doi: 10.1073/pnas.86.8.2863
11. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature.* (1990) 348:730–2. doi: 10.1038/348730a0
12. Rayhman O, Klipper E, Muller L, Davidson B, Reich R, Meidan R. Small interfering RNA molecules targeting endothelin-converting enzyme-1 inhibit endothelin-1 synthesis and the invasive phenotype of ovarian carcinoma cells. *Cancer Res.* (2008) 68:9265–73. doi: 10.1158/0008-5472.CAN-08-2093
13. Houde M, Desbiens L, D'Orléans-Juste P. Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol.* (2016) 77:143–75. doi: 10.1016/bs.apha.2016.05.002
14. Vacca F, Bagnato A, Catt KJ, Tecce R. Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res.* (2000) 60:5310–7.
15. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci.* (2002) 115:455–65.
16. Zhou XE, Melcher K, Xu HE. Understanding the GPCR biased signaling through G protein and arrestin complex structures. *Curr Opin Struct Biol.* (2017) 45:150–9. doi: 10.1016/j.sbi.2017.05.004
17. Maguire JJ, Kuc RE, Pell VR, Green A, Brown M, Kumar S, et al. Comparison of human ETA and ETB receptor signalling via G-protein and β-arrestin pathways. *Life Sci.* (2012) 91:544–9. doi: 10.1016/j.lfs.2012.03.021
18. Peterson YK, Luttrell LM. The diverse roles of arrestin scaffolds in G-protein-coupled receptor signaling. *Pharmacol Rev.* (2017) 69:256–97. doi: 10.1124/pr.116.013367
19. Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, Attramadal H. Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem.* (2000) 275:17596–604. doi: 10.1074/jbc.M000142200
20. Ranjan R, Dwivedi H, Baidya M, Kumar M, Shukla AK. Novel structural insights into GPCR-β-arrestin interaction and signaling. *Trends Cell Biol.* (2017) 27:851–62. doi: 10.1016/j.tcb.2017.05.008
21. Rosanò L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Spadaro F, et al. β-arrestin-1 is a nuclear transcriptional regulator of endothelin-1-induced β-catenin signaling. *Oncogene.* (2013) 32:5066–77. doi: 10.1038/onc.2012.527
22. Rosanò L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Caprara V, et al. Endothelin A receptor/β-arrestin signaling to the Wnt pathway renders ovarian cancer cells resistant to chemotherapy. *Cancer Res.* (2014) 74:7453–64. doi: 10.1158/0008-5472.CAN-13-3133
23. Cianfrocca R, Tocci P, Rosanò L, Caprara V, Sestito R, Di Castro V, et al. Nuclear β-arrestin1 is a critical cofactor of hypoxia-inducible factor-1α signaling in endothelin-1-induced ovarian tumor progression. *Oncotarget.* (2016) 7:17790–804. doi: 10.18632/oncotarget.7461

24. Cianfrocca R, Tocci P, Semprucci E, Spinella F, Di Castro V, Bagnato A, et al. β-arrestin 1 is required for endothelin-1-induced NF-κB activation in ovarian cancer cells. *Life Sci.* (2014) 118:179–84. doi: 10.1016/j.lfs.2014.01.078
25. Semprucci E, Tocci P, Cianfrocca R, Sestito R, Caprara V, Veglione M, et al. Endothelin A receptor drives invadopodia function and cell motility through the β-arrestin/PDZ-RhoGEF pathway in ovarian carcinoma. *Oncogene.* (2015) 35:3432–42. doi: 10.1038/onc.2015.403
26. Di Modugno F, Caprara V, Chellini L, Tocci P, Spadaro F, Ferrandina G, et al. hMENA is a key regulator in endothelin-1/β-arrestin1-induced invadopodial function and metastatic process. *Proc Natl Acad Sci USA.* (2018) 115:3132–7. doi: 10.1073/pnas.1715998115
27. Chellini L, Caprara V, Spadaro F, Sestito R, Bagnato A, Rosanò L. Regulation of extracellular matrix degradation and metastatic spread by IQGAP1 through endothelin-1 receptor signaling in ovarian cancer. *Matrix Biol.* (2018) 81:17–33. doi: 10.1016/j.matbio.2018.10.005
28. Crudden C, Shibano T, Song D, Suleymanova N, Girnita A, Girnita L. Blurring boundaries: receptor tyrosine kinases as functional G protein-coupled receptors. *Int Rev Cell Mol Biol.* (2018) 339:1–40. doi: 10.1016/bs.ircmb.2018.02.006
29. Min J, Defea K. β-arrestin-dependent actin reorganization: bringing the right players together at the leading edge. *Mol Pharmacol.* (2011) 80:760–8. doi: 10.1124/mol.111.072470
30. Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, et al. Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. *Proc Natl Acad Sci USA.* (2009) 106:2806–11. doi: 10.1073/pnas.0807158106
31. Zecchini V, Madhu B, Russell R, Pértega-Gomes N, Warren A, Gaude E, et al. Nuclear ARRB1 induces pseudohypoxia and cellular metabolism reprogramming in prostate cancer. *EMBO J.* (2014) 33:1365–82. doi: 10.15252/embj.201386874
32. Shenoy SK, Han S, Zhao YL, Hara MR, Oliver T, Cao Y, et al. (2012). β-arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene.* (2012) 31:282–92. doi: 10.1038/onc.2011.238
33. Tocci P, Cianfrocca R, Di Castro V, Rosanò L, Sacconi A, Donzelli S, et al. β-arrestin1/YAP/mutant p53 complexes orchestrate the endothelin A receptor signaling in high-grade serous ovarian cancer. *Nat Commun.* (2019)10:3196. doi: 10.1038/s41467-019-11045-8
34. Moroishi T, Hansen CG, Guan KL. The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer.* (2015) 15:73–9. doi: 10.1038/nrc3876
35. Totaro A, Panciera T, Piccolo S. YAP/TAZ upstream signals and downstream responses. *Nat Cell Biol.* (2018) 20:888–99. doi: 10.1038/s41556-018-0142-z
36. Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.* (2008) 22:1962–71. doi: 10.1101/gad.1664408
37. Di Agostino S, Sorrentino G, Ingallina E, Valenti F, Ferraiuolo M, Bicciato S, et al. YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins. *EMBO Rep.* (2016) 17:188–201. doi: 10.15252/embr.201540488
38. Huang WH, Chang MC, Tsai KS, Hung MC, Chen HL, Hung SC. Mesenchymal stem cells promote growth and angiogenesis of tumors in mice. *Oncogene.* (2013) 32:4343–54. doi: 10.1038/onc.2012.458
39. Hinsley EE, Hunt S, Hunter KD, Whawell SA, Lambert DW. Endothelin-1 stimulates motility of head and neck squamous carcinoma cells by promoting stromal-epithelial interactions. *Int J Cancer.* (2012) 130:40–7. doi: 10.1002/ijc.25968
40. Moraitis S, Miller WR, Smyth JF, Langdon SP. Paracrine regulation of ovarian cancer by endothelin. *Eur J Cancer.* (1999) 35:1381–7.
41. Gurulli G, Pflug BR, Pecher S, Makarenkova V, Shurin MR, Nelson JB. Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops. *Blood.* (2004) 104:2107–15. doi: 10.1182/blood-2003-10-3559
42. Kandalaft LE, Motz GT, Duraiswamy J, Coukos G. Tumor immune surveillance and ovarian cancer: lessons on immune mediated tumor rejection or tolerance. *Cancer Metastasis Rev.* (2011) 30:141–51. doi: 10.1007/s10555-011-9289-9
43. Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G. Endothelin B receptor, a new target in cancer immune therapy. *Clin Cancer Res.* (2009) 15:4521–8. doi: 10.1158/1078-0432.CCR-08-0543
44. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat Med.* (2008) 14:28–36. doi: 10.1038/nm1699
45. Morand J, Briançon-Marjollet A, Lemarie E, Gonthier B, Arnaud J, Korichneva I, et al. Zinc deficiency promotes endothelin secretion and endothelial cell migration through nuclear hypoxia-inducible factor-1 translocation. *Am J Physiol Cell Physiol.* (2019) 317:C270–6. doi: 10.1152/ajpcell.00460.2018
46. Spinella F, Garrafa E, Di Castro V, Rosanò L, Nicotra MR, Caruso A, et al. Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. *Cancer Res.* (2009) 69:28–36. doi: 10.1158/0008-5472.CAN-08-1879
47. Ma Z, Yu YR, Badea CT, Kovacs JJ, Xiong X, Comhair S, et al. Vascular endothelial growth factor receptor 3 regulates endothelial function through β-arrestin 1. *Circulation.* (2019) 139:1629–42. doi: 10.1161/CIRCULATIONAHA.118.034961
48. Gutkind JS, Kostenis E. Arrestins as rheostats of GPCR signalling. *Nat Rev Mol Cell Biol.* (2018) 19:615–16. doi: 10.1038/s41580-018-0041-y
49. Smith JS, Lefkowitz, RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov.* (2018) 17:243–60. doi: 10.1038/nrd.2017.229
50. Xiao K, Sun J. Elucidating structural and molecular mechanisms of β-arrestin-biased agonism at GPCRs via MS-based proteomics. *Cell Signal.* (2018) 41:56–64. doi: 10.1016/j.cellsig.2017.09.013
51. Davenport AP, Kuc RE, Southan C, Maguire JJ. New drugs and emerging therapeutic targets in the endothelin signaling pathway and prospects for personalized precision medicine. *Physiol Res.* (2018) 67:S37–54.
52. Kim SJ, Kim JS, Kim SW, Brantley E, Yun SJ, He J, et al. Macitentan (ACT-064992), a tissue-targeting endothelin receptor antagonist, enhances therapeutic efficacy of paclitaxel by modulating survival pathways in orthotopic models of metastatic human ovarian cancer. *Neoplasia.* (2011) 13:167–79. doi: 10.1593/neo.10806
53. Kim SJ, Kim JS, Kim SW, Yun SJ, He J, Brantley E, et al. Antivascular therapy for multidrug-resistant ovarian tumors by macitentan, a dual endothelin receptor antagonist. *Transl Oncol.* (2012) 5:39–47. doi: 10.1593/tlo.11286
54. Kim SJ, Lee HJ, Kim MS, Choi HJ, He J, Wu Q, et al. Macitentan, a dual endothelin receptor antagonist, in combination with temozolomide leads to glioblastoma regression and long-term survival in mice. *Clin Cancer Res.* (2015) 21:4630–41. doi: 10.1158/1078-0432.CCR-14-3195
55. Lee HJ, Hanibuchi M, Kim SJ, Yu H, Kim MS, He J, et al. Treatment of experimental human breast cancer and lung cancer brain metastases in mice by macitentan, a dual antagonist of endothelin receptors, combined with paclitaxel. *Neuro Oncol.* (2016) 18:486–96. doi: 10.1093/neuonc/now037
56. Askoxylakis V, Ferraro GB, Badeaux M, Kodack DP, Kirst I, Shankaraiah RC, et al. Dual endothelin receptor inhibition enhances T-DM1 efficacy in brain metastases from HER2-positive breast cancer. *NPJ Breast Cancer.* (2019) 5:4. doi: 10.1038/s41523-018-0100-8
57. Dhaun N, Webb DJ. Endothelins in cardiovascular biology and therapeutics. *Nat Rev Cardiol.* (2019) 16:491–502. doi: 10.1038/s41569-019-0176-3
58. US National Library of Medicine. *ClinicalTrials.gov*. (2019). Available online at: https://ClinicalTrials.gov/ct2/show/NCT02603809
59. Magdeldin T, López-Dávila V, Pape J, Cameron GW, Emberton M, Loizidou M, et al. Engineering a vascularised 3D *in vitro* model of cancer progression. *Sci Rep.* (2017) 7:44045. doi: 10.1038/srep44045

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 25 October 2019
doi: 10.3389/fendo.2019.00714

Multifactorial Regulation of Myometrial Contractility During Pregnancy and Parturition

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The steroid hormones progesterone (P_4) and estradiol-17β (E_2), produced by the placenta in humans and the ovaries in rodents, serve crucial roles in the maintenance of pregnancy, and the initiation of parturition. Because of their critical importance for species survival, the mechanisms whereby P_4 and its nuclear receptor (PR) maintain myometrial quiescence during pregnancy, and for the decline in P_4/PR and increase in E_2/estrogen receptor (ER) function leading to parturition, are multifaceted, cooperative, and redundant. These actions of P_4/PR include: (1) PR interaction with proinflammatory transcription factors, nuclear factor κB (NF-κB), and activating protein 1 (AP-1) bound to promoters of proinflammatory and contractile/contraction-associated protein (CAP) genes and recruitment of corepressors to inhibit NF-κB and AP-1 activation of gene expression; (2) upregulation of inhibitors of proinflammatory transcription factor activation (IκBα, MKP-1); (3) induction of transcriptional repressors of CAP genes (e.g., ZEB1). In rodents and most other mammals, circulating maternal P_4 levels remain elevated throughout most of pregnancy and decline precipitously near term. By contrast, in humans, circulating P_4 levels and myometrial PR levels remain elevated throughout pregnancy and into labor. However, even in rodents, wherein P_4 levels decline near term, P_4 levels remain higher than the K_d for PR binding. Thus, parturition is initiated in all species by a series of molecular events that antagonize the P_4/PR maintenance of uterine quiescence. These events include: direct interaction of inflammatory transcription factors (e.g., NF-κB, AP-1) with PR; increased expression of P_4 metabolizing enzymes; increased expression of truncated/inhibitory PR isoforms; altered expression of PR coactivators and corepressors. This article will review various mechanisms whereby P_4 acting through PR isoforms maintains myometrial quiescence during pregnancy as well as those that underlie the decline in PR function leading to labor. The roles of P_4- and E_2-regulated miRNAs in the regulation and integration of these mechanisms will also be considered.

Keywords: progesterone, gene regulation, transcription corepressor, inflammation, pregnancy, myometrium, NF-κB

OPEN ACCESS

Edited by:
Tamas Balla, National Institutes of Health (NIH), United States

Reviewed by:
Dragos Cretoiu, Carol Davila University of Medicine and Pharmacy, Romania
Tamas Zakar, University of Newcastle, Australia

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *10 July 2019*
Accepted: *03 October 2019*
Published: *25 October 2019*

Citation:
Mendelson CR, Gao L and Montalbano AP (2019) Multifactorial Regulation of Myometrial Contractility During Pregnancy and Parturition. Front. Endocrinol. 10:714. doi: 10.3389/fendo.2019.00714

INTRODUCTION

Preterm birth (<37 weeks gestation), which affects ~15 × 10^6 births globally each year, is a major cause of death within the first month of postnatal life (1). The highest rates of preterm birth (≥15% of all live births) occur in sub-Saharan Africa, Pakistan, and Indonesia. In the U.S., the preterm birth rate remains at ~10% of all overall live births. However, significant

racial disparities in preterm birth rates exist, with the incidence of preterm birth among African-Americans being 50% higher than that of the overall population. Notably, the underlying causes for these racial differences remain unknown (2). Astonishingly, the modalities used to treat and/or prevent preterm labor have changed little over the past 50 years. This is due, in part, to our incomplete comprehension of mechanisms that mediate myometrial quiescence and contractility as well as the reluctance of pharmaceutical companies to engage in drug discovery in this critical area.

Throughout pregnancy, myometrial quiescence is controlled by increased progesterone (P_4), secreted by the placenta and/or the ovarian corpus luteum, depending upon the species. In humans, two progesterone receptor (PR) isoforms, PR-A (94 kDa), and PR-B (114 kDa), alternative transcripts of a single gene (3, 4), mediate P_4 action to block myometrial contractility. Both PR-A and PR-B bind to progesterone response elements (PREs) in DNA; however, PR-A contains two of three transcriptional activation domains that are present in PR-B and is, therefore, less transcriptionally active. Thus, PR-A can repress PR-B transcriptional activity in a cell- and gene-specific context (5, 6). PR-A was also found to inhibit PR-B transcriptional activity in cultured human myometrial cells (7), suggesting a potential antagonistic role of PR-A on PR-B action in the myometrium. PR-A and PR-B are differentially regulated in the human myometrium during pregnancy (8); the ratio of PR-A to PR-B mRNA (9) and protein (7) was observed to increase significantly in the myometrium of women in labor when compared to those not in labor at term. In telomerase-immortalized human myometrial (hTERT-HM) cells stably expressing either PR-A or PR-B, P_4 treatment had increased anti-inflammatory activity in PR-B-expressing cells when compared to those expressing PR-A (10).

As described below, a number of unique and redundant mechanisms mediate the action of P_4/PR to maintain uterine quiescence. In rodents and most other mammals, circulating maternal P_4 levels remain elevated throughout most of pregnancy and decline sharply prior to parturition (11). This has led to the concept that labor is associated with P_4 withdrawal. By contrast, in humans and guinea pigs circulating P_4 and myometrial levels of PR fail to decline during late pregnancy and into labor (12). However, treatment with PR antagonists can cause increased myometrial contractility, cervical ripening, and/or increased sensitivity to labor induction by contractile factors (13–16). Importantly, even in rodents, circulating maternal P_4 levels at term remain well above the equilibrium dissociation constant for binding to PR (17). Moreover, in rodents, local metabolism of P_4 within the cervix and myometrium to inactive products near term is essential for the normal timing of parturition. Thus, in mice deficient in 5α-reductase type I (expressed in cervix) (18, 19) or 20α-hydroxysteroid dehydrogenase (20α-HSD, expressed in myometrium) (20) parturition is severely delayed. Collectively, these findings have led to the concept that parturition in all placental mammals is initiated by a conserved sequence of molecular events that impairs the capacity of the PR to maintain uterine quiescence. These include: (1) direct interaction of transcription factor nuclear factor κB (NF-κB) with PR; (2) upregulation of P_4 metabolizing enzymes within the uterus and cervix; (3) increased expression of truncated/inhibitory PR isoforms; (4) altered expression of key PR-interacting coactivators and corepressors. This article will review the various mechanisms whereby P_4 acting through PR isoforms maintains myometrial quiescence during pregnancy as well as those that underlie the decline in PR function leading to parturition.

PARTURITION IS ASSOCIATED WITH AN INCREASED INFLAMMATORY RESPONSE

Term and preterm parturition is initiated by an enhanced inflammatory response, increased levels of proinflammatory cytokines in amniotic fluid (21) and the invasion of the fetal membranes, cervix and myometrium by neutrophils and macrophages (Mϕ) (22–24) (**Figure 1**). The secretion of cytokines and chemokines by the invading immune cells (26) cause activation of NF-κB and other inflammation-associated transcription factors (e.g., AP-1) (23, 27–30). These activated transcription factors promote increased expression of myometrial proinflammatory [e.g., interleukin (*IL*)-*1β, IL-8*] and contractile/CAP [connexin-43 (*CX43/GJA1*), oxytocin receptor (*OXTR*), and cyclooxygenase 2 (*COX-2/PTGS2*)] genes, leading to parturition (31–34). Whereas, intra-amniotic infection associated with chorioamnionitis can provide the stimulus for the inflammatory response leading to preterm labor (35), signals from both mother and fetus provide critical inflammatory stimuli leading to labor at term.

Increased Mechanical Stretch of the Uterus Results in Production of Chemokines, Enhanced Immune Cell Invasion, and Proinflammatory Signaling Leading to Labor

Near term, enhanced uterine stretch caused by the growing fetus provides an important stimulus for the initiation of labor (36, 37) (**Figure 1**). The increased incidence of preterm birth in twin and multiple, as compared to singleton, pregnancies implicates uterine over-distension as a causative factor (38). The expression of the β-chemokine, monocyte chemoattractant protein-1/C-C motif ligand 2 (MCP-1/CCL2), which attracts and activates Mϕ, was found to be upregulated in the term pregnant myometrium of women in labor, as compared to myometrium from women not in labor (39) and of pregnant rats prior to and during parturition (36). In pregnant rats carrying pups only in one uterine horn, increased MCP-1 expression was observed only in the gravid horn, implying the potential role of the fetus and/or of uterine stretch (36) in the induction of MCP-1 expression. Furthermore, the findings that MCP1 expression and Mϕ infiltration were greatly increased in the pregnant rat uterus with preterm labor induction by the PR antagonist, mifepristone/RU486, and inhibited by progestin treatment to delay parturition (36) suggests a role of P_4/PR in MCP-1 regulation. Preterm labor

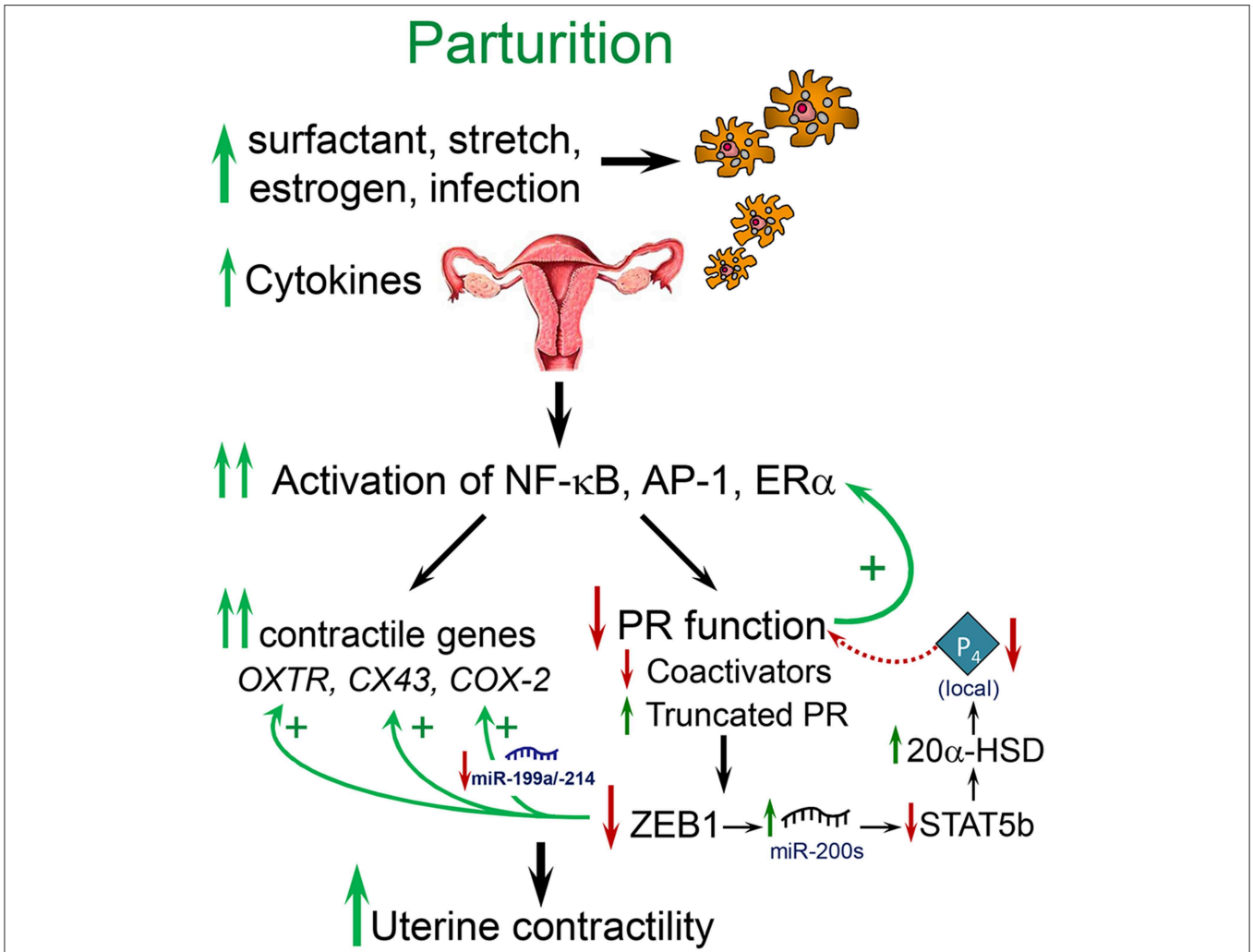

FIGURE 1 | Labor at term or preterm is associated with an inflammatory response. Near term, signals from the fetus (e.g., increased surfactant lipoprotein secretion, increased placental CRH, cortisol, and estrogen) and mother (e.g., uterine stretch with increased chemokine production, increased estrogen receptor signaling), cause increased immune cell invasion of the fetal membranes, decidua, and myometrium. At preterm, immune cell invasion is stimulated by ascending infection associated with chorioamnionitis as well as increased uterine stretch in twin or multi-fetal pregnancies. The invading immune cells produce proinflammatory cytokines that cause activation of inflammatory transcription factors, such as NF-κB and AP-1, which promote activation of proinflammatory and *CAP* gene expression and a decline in PR function, caused by a decrease in coactivators and increased expression of PR-A and other truncated PR isoforms. The decline in PR function results in decreased ZEB1 expression, with increased expression of contractile genes (*OXTR* and *CX43*) and miR-200 family members and decreased expression of the miR-199a/214 cluster. The increase in miR-200s suppress their targets ZEB1/2 and STAT5b and increase local P_4 metabolism via upregulation of 20α-HSD. The decrease in miR-199a/214 results in upregulation of their target, COX-2, and an increase in synthesis of contractile prostaglandins. These events culminate in an increase in myometrial contractility leading to parturition. Modified from Mendelson et al. (25).

was induced in non-human primates by intrauterine balloon inflation, in association with increased expression of IL-6, IL-8, and CCL-2 in the myometrium (40). Thus, enhanced inflammation associated with mechanical stress contributes to the initiation of term and preterm labor. In studies using human myometrial smooth muscle cells in culture, IL-1β and TNFα induced expression of MCP-1 and other chemokines; this was blocked by an inhibitor of the NF-κB signaling pathway (41). Likewise, in human choriodecidual and breast cancer cells, MCP-1 was stimulated by NF-κB activation and inhibited by P_4/PR (42).

Increased Estrogen Receptor Signaling Contributes to the Inflammatory Response Leading to Parturition

Across mammalian species, an increase in circulating estradiol-17β (E_2) (43, 44) and/or myometrial estrogen receptor α (ERα) activity (9, 45) precedes the increase in uterine contractility near term (**Figure 1**). Estrogens induce migration of immune cells to the uterus and antagonize anti-inflammatory actions of P_4/PR (9, 46). Moreover, ERα activation enhances transcription of the *CAP* genes, *OXTR* (47), *CX43* (48), and *COX-2* (9), and the resulting synthesis of prostaglandins that increase myometrial contractility

(49–51). These actions of estrogen may be mediated, in part, through interaction of ERα and p160 coactivators with the AP-1 transcription factors Fos and Jun at AP-1-regulated promoters, resulting in an increase in AP-1 transcriptional activity (52).

Interestingly, we observed that ERα is a direct target of the microRNA, miR-181a, which significantly declines in mouse myometrium near term and in term myometrial tissues from women in labor, compared to those not-in-labor (53). Furthermore, E_2 treatment inhibited miR-181a expression in uteri of ovariectomized mice and in human myometrial cells in primary culture. This revealed the presence of a feedback loop, wherein increased circulating E_2 near term causes suppression of miR-181a, resulting in upregulation of ERα with further downregulation of miR-181a (53). In human myometrial cells, overexpression of miR-181a mimics repressed TNFα, CCL-2 and CCL-8 expression, while expression of the anti-inflammatory cytokine, IL-10, increased (53). TNFα was confirmed as a direct target of miR-181a, while CCL-2 and CCL-8 are predicted targets of this miRNA (53). c-Fos, which increases in pregnant rat (54) and mouse (53) myometrium during late gestation and into labor, was validated as a target of miR-181a in dendritic cells (55). These collective findings suggest that, from early through mid-gestation, relatively low E_2/ERα levels allow increased expression miR-181a in myometrium, which represses ERα, c-FOS, TNFα, and several other proinflammatory cytokines, and increases the expression of anti-inflammatory cytokines. Moreover, near term increased circulating levels of E_2 inhibit miR-181a, which allows the upregulation of its targets, ERα, TNFα, other proinflammatory cytokines, and transcription factor, c-FOS. In turn, c-FOS mediates the proinflammatory effects of E_2/ERα and cytokines, which activate *CAP* genes and lead to labor.

We also previously observed that in concert with the increased expression of the miR-200 family in pregnant mouse myometrium between 15.5 days post-coitum (dpc) and term (18.5 dpc and in labor) (56), there was a decline in the expression of the miR-199a/miR-214 cluster of miRNAs (57) (**Figure 2**). This was mediated by increased E_2/ERα and the decrease in PR function, which inhibited expression of transcription factor ZEB1, a positive regulator of *miR-199a/miR-214* transcription (57, 58). Of note, miR-199a-3p and miR-214 directly target COX-2, which increases in the myometrium near term and during labor. Therefore, stimulatory effects of E_2 on COX-2 expression (50) are likely mediated, in part, by its inhibition of miR-199a-3p/miR-214. Since miR-181a targets both ERα and cFOS (53), we suggest that the coordinate decline in miR-181a and miR-199a-3p/214 in the myometrium toward term mediates the induction of COX-2 expression via indirect and direct mechanisms.

The Fetus Produces Signaling Molecules for the Initiation of Parturition

The fetus has been proposed to contribute to the initiation of parturition through the production of signaling molecules from its adrenals, placenta and lungs.

Cortisol Production by the Fetal Adrenal

In sheep, increased cortisol production by the fetal adrenal has been implicated in the initiation of parturition via the activation of placental COX-2 and production of prostaglandins (59). The prostaglandins, in turn, stimulate 17α-hydroxylase/17,20 lyase (CYP17) expression, resulting in increased placental production of C_{19}-steroids, which are metabolized to estrogens by placental aromatase P450 (CYP19). The increased estrogens may antagonize PR function (12) and upregulate *CAP* gene expression via the mechanisms described above. The surge of fetal cortisol also promotes fetal lung maturation and synthesis of surfactant components (60), which, as described below, also serve as fetal signals for the initiation of labor.

Corticotropin-Releasing Hormone (CRH)

In humans, the placenta lacks the capacity to express *CYP17* and produce C_{19}-steroids, which are instead synthesized in large quantities by the fetal adrenals (61). However, the human placenta is unique in its ability to secrete CRH (62), which is produced in increasing amounts near term and has been suggested to provide a fetal signal for the initiation of parturition (63, 64). Fetal placental CRH is proposed to upregulate secretion of adrenocorticotropic hormone (ACTH) by the fetal pituitary, which stimulates production of cortisol and the C_{19}-steroid, dehydroepiandrosterone sulfate (DHEAS), by the fetal adrenals. DHEAS is subsequently metabolized within the placenta to estrogens, which, as mentioned, mediate inflammatory signaling leading to labor. CRH mRNA is also expressed at high levels in the bronchiolar epithelium of 13.5–17.5 dpc fetal mouse lung (65). It has been suggested that CRH may act directly within the fetal lung to promote synthesis of the major surfactant protein, surfactant protein (SP)-A. Accordingly, in *CRH* null mouse fetuses of *CRH*-deficient mothers, lung maturation and induction of *SP-A* expression were found to be delayed (66). Thus, CRH may act directly within the fetal lung to stimulate the production of surfactant components, and/or to increase fetal ACTH and adrenal cortisol production to enhance fetal lung development and surfactant synthesis. Accordingly, as described below, augmented surfactant production by the maturing fetal lung likely serves as an important fetal signal for the initiation of labor.

Surfactant Components Secreted by the Fetal Lung

Increased production of pulmonary surfactant components by the maturing fetal lung is proposed to signal the initiation of parturition (23, 67–70). Lung surfactant is a glycerophospholipid-rich surface-active lipoprotein produced specifically by type II cells of the pulmonary alveoli, which acts to reduce surface tension at the alveolar air-liquid interface after birth. Surfactant synthesis by the developing lung is initiated after ∼85% of gestation is complete. Consequently, premature infants born prior to this time are at risk of developing respiratory distress syndrome due to surfactant deficiency. Dipalmitoylphosphatidylcholine (DPPC) is the major surfactant glycerophospholipid and most surface-active component. Approximately 10% of surfactant composition is comprised of the essentially lung-specific proteins SP-A, SP-B, SP-C, and

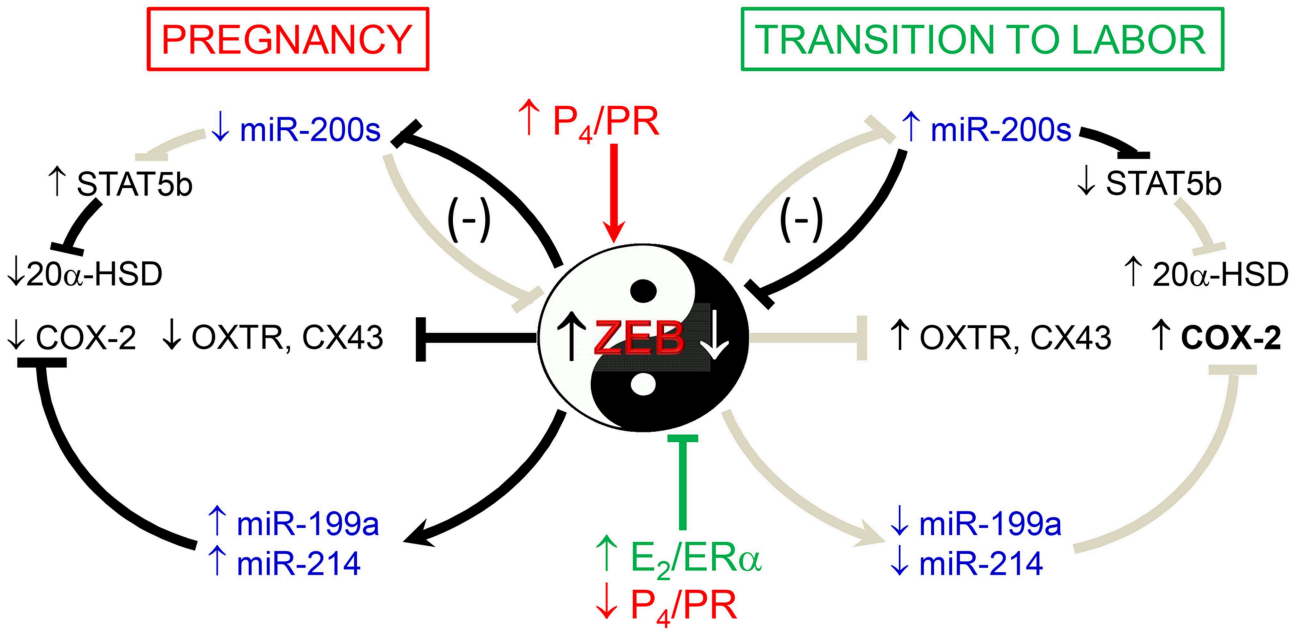

FIGURE 2 | Opposing actions of P_4 and E_2 on myometrial contractility during pregnancy and labor are mediated by ZEB1 and ZEB2 and miRNAs. During pregnancy, increased P_4/PR function causes the induction of ZEB1, which in turn inhibits expression of the miR-200 family and *CAP* genes and enhances expression of miR-199a-3p and miR-214, which cause suppression of their target, COX-2. Decreased levels of miR-200 family members cause a further increase in ZEB1 and enhance ZEB2 as well as STAT5b, which suppresses 20α-HSD expression to maintain increased tissue levels of P_4. During the transition to labor, the decrease in P_4/PR and increase in E_2/ER function cause a decline in ZEB1. This allows for the upregulation of miR-200 family expression, causing further suppression of ZEB1 and inhibition of ZEB2 as well as STAT5b. The decrease in STAT5b allows upregulation of 20α-HSD and increased local metabolism of P_4 to inactive products, further reducing PR function. The decline in ZEB1/2 allows upregulation of *CAP* genes and causes a decrease in *miR-199a/-214* expression, which allows for the induction of COX-2 and production of contractile prostaglandins. Collectively, these events culminate in parturition. Reproduced from Renthal et al. (58).

SP-D (71, 72). SP-B and SP-C are lipophilic peptides produced from larger precursors. SP-B serves an essential role, together with DPPC, in the reduction of alveolar surface tension (71, 73). SP-A and SP-D serve as C-type lectin components of the innate immune system (74) that enhance the uptake of a variety of microbes by Mϕ (74–77).

The role of surfactant components in the initiation of parturition was first suggested by the finding that surfactant isolated from human amniotic fluid stimulated prostaglandin synthesis in amnion discs (67). It was proposed that amniotic fluid surfactant phospholipids provide a source of arachidonic acid as a substrate for synthesis of contractile prostaglandins. Others suggested that a substance in human amniotic fluid secreted in urine from the fetal kidney enhanced PGE_2 production by human amnion cells (78); however, this amniotic fluid "substance" is likely derived from the fetal lung. Accordingly, Johnston and colleagues (79) suggested that platelet-activating factor (PAF), a potent proinflammatory phospholipid secreted into amniotic fluid with fetal lung surfactant near term, may enhance myometrial contractility leading to labor. Our laboratory has obtained extensive evidence for the roles of fetal lung SP-A and PAF as key fetus-derived inflammatory signals for the initiation of parturition (23, 69, 70).

SP-A and Toll-like receptor 2 (TLR2)

In all species studied, synthesis of SP-A by the fetal lung is developmentally upregulated with surfactant glycerophospholipids after ∼85% of gestation is complete (80). Consequently, SP-A serves as a relevant marker of fetal lung maturity and surfactant production. SP-A expression in mouse fetal lung and its secretion into amniotic fluid are upregulated at 17.5 dpc and continue to increase toward term (19.5 dpc) (23, 81, 82). This is temporally associated with increased proinflammatory cytokine production by amniotic fluid Mϕ, their migration to the maternal uterus and the activation of uterine NF-κB (23). In studies using *Rosa 26 Lac-Z* mice, we observed that fetal Mϕ migrated to the maternal uterus with the induction in SP-A expression by the fetal lung during late gestation. Moreover, an intra-amniotic injection of SP-A caused preterm delivery of fetuses, and was associated with the activation of uterine NF-κB within 4.5 h. Conversely, injection of an SP-A antibody or NF-κB inhibitor into amniotic fluid delayed labor by >24 h (23). These findings suggested that enhanced SP-A secretion by the fetal lung near term causes activation and migration of fetal AF Mϕ to the maternal uterus, where increased cytokine production activates NF-κB and a signaling cascade, leading to labor. It should be noted that studies using laser capture of limited numbers of $CD68^+$ or $CD14^+$ (Mϕ markers) cells from the superficial portion of the myometrium from women carrying a male fetus at term failed to identify fetal mononuclear cells (83). Moreover, incubation of human amnion disks with SP-A resulted in upregulation of anti-inflammatory cytokines and cytokine receptors (84). However, SP-A can have both pro- and anti-inflammatory actions depending upon

the cellular environment, as well as the receptor to which it binds (85).

To further study the roles of SP-A, the related C-type lectin, SP-D, and their putative receptor, TLR2 (86–89), in the initiation of parturition, we utilized gene-targeted mice. In first pregnancies, *SP-A*$^{-/-}$ and *SP-A*$^{-/-}$/*SP-D*$^{-/-}$ female mice bred to genetically like males delivered at term (19.5 dpc). However, in subsequent pregnancies, these gene-targeted mice manifested a ∼12 h delay in parturition, associated with significantly reduced levels of myometrial Cx43, Oxtr, IL-1β, and IL-6 mRNA at 18.5 dpc compared to wild-type (WT) mice (69). We postulated that the parturition timing difference in the deficient mice in first vs. second pregnancies was due to the dominant role of uterine mechanical stretch as a signal for parturition (36, 37, 90) in first pregnancies. However, in subsequent pregnancies, prior adaptation of the uterus to stretch (91) may allow other signals (e.g., surfactant proteins) to play a more significant role. *TLR2*$^{-/-}$ females manifested a significant delay (∼12 h) in parturition timing during first pregnancies, as well as reduced expression of *CAP* genes and the Mϕ marker, F4/80, in myometrium at term compared to WT (69). F4/80^{+} AF Mϕs from *TLR2*$^{-/-}$ and *SP-A/D*$^{-/-}$ mice expressed significantly lower levels of both pro-inflammatory and anti-inflammatory activation markers, compared to those of gestation-matched WT mice (69). These findings suggested that SP-A and SP-D act via TLR2 on fetal-derived Mϕ to modulate parturition timing; their impact may depend upon parity.

Roles of SRC-1 and SRC-2 in production of fetal signals leading to labor

Previously, we observed that the p160 family members (92), steroid receptor coactivators, SRC-1, and SRC-2/TIF-2, are critical for transcriptional upregulation of *SP-A* gene expression in fetal lung type II cells (93–95). SRCs do not bind to DNA directly; however they regulate gene transcription by interacting with steroid receptors and other transcription factors and by recruiting other coregulators with histone-modifying activities (92, 96) to alter chromatin structure (97). Notably, gene-targeted mice that are singly-deficient in *Src-1, Src-2,* or *Src-3* manifest various reproductive phenotypes (96). Importantly, mice that were double-knockout (dKO) for *Src-1* and *Src-2* died at birth from respiratory distress (98), which is indicative of lung surfactant deficiency. This observation was of great interest to us, considering the critical roles of SRC-1 (94) and SRC-2 (93) in *SP-A* expression.

To characterize these mice further, we crossed *Src-1*$^{+/-}$/*Src-2*$^{+/-}$ (*Src-1/-2 dhet*) males and females. Remarkably, they manifested severely delayed parturition (∼38 h). This parturition delay occurred with significant reductions in NF-κB activation, as well as decreased expression of *Oxtr*, *Cx43, PGF*$_2\alpha$ *synthase/Akr1b3* and levels of the contractile prostaglandin, $PGF_2\alpha$, in the maternal myometrium. The decrease in myometrial $PGF_2\alpha$ was associated with impaired luteolysis and elevated circulating P_4 (70). Notably, parturition timing was normal in *Src-1-KO* females and in *Src-2*$^{+/-}$ females bred to genetically like males, revealing that *Src-1/-2* double-deficiency is requisite for the delay in parturition. Importantly, WT females bred to *Src-1/-2* double-deficient males exhibited a parturition delay equivalent to that observed in our crosses of *Src-1/-2* double-deficient males and females, with decreased myometrial NF-κB activation and *CAP* gene expression and elevated circulating P_4. These findings indicated that the defect responsible for delayed parturition with *Src-1/-2* double deficiency was fetal in origin. Because of the importance of fetal lung SP-A production in the timing of parturition, it was of great interest that SP-A levels were significantly reduced in the lungs and amniotic fluid of fetuses doubly deficient in Src-1 and Src-2 when compared to WT. On the other hand, levels of SP-A in lungs and amniotic fluid of Src-1 or Src-2 singly deficient fetuses were similar to WT.

Clearly, the increase in gestation length in mice carrying *Src-1/-2* doubly deficient fetuses (∼38 h) was significantly greater than what we observed in *SP-A*-deficient and in *SP-A/SP-D* double-deficient mice (∼12 h). This suggested that signaling molecules, other than SP-A and SP-D, were affected by double-deficiency of *Src-1* and *Src-2*. As noted, *Src-1/-2 dKO* mice succumbed at birth to alveolar collapse/atelectasis (98), and this suggested that surfactant glycerophospholipids may also be altered. In this regard, we found that amniotic fluid levels of the major and most surface-active surfactant component, DPPC, were significantly reduced in *Src-1/-2 dKO* fetuses compared to WT (70).

We also considered the role of the glycerophospholipid, PAF, which is proinflammatory, produced by the developing fetal lung together with surfactant lipids and SP-A and secreted into amniotic fluid near term. PAF, which activates leukocytes and stimulates their migration, was suggested to contribute to the initiation of term and preterm labor (68, 79, 99–101). PAF also directly stimulated the contraction of myometrial strips (102–106). Intriguingly, we observed that PAF levels in fetal lungs and amniotic fluid of *SRC-1/-2* double-deficient mice failed to increase toward term and were significantly reduced, compared to WT fetuses or those singly deficient in *Src-1* or *Src-2*.

Since both DPPC and PAF levels were significantly decreased in the amniotic fluid of *Src-1/-2* double-deficient fetuses, we searched for glycerophospholipid metabolizing enzymes that might coordinately regulate the synthesis of both of these molecules. In so doing, we discovered that lysophosphatidylcholine acyltransferase 1 (Lpcat1), which serves a key role in the deacylation/reacylation of the *sn-2* position of both DPPC and PAF to make the surface-active and pro-inflammatory molecules, respectively (107–109), was significantly decreased in lungs of *Src-1/-2* double-deficient fetuses compared to WT (70). Lpcat1 was previously found to be expressed specifically in mouse lung type II cells, developmentally-induced in fetal lung toward term, and stimulated by glucocorticosteroids (108). The role of Lpcat1 in surfactant synthesis by the developing lung was supported by the finding that mice carrying a hypomorphic allele of *Lpcat1* manifested atelectasis at birth and a deficiency in surfactant DPPC (107). Notably, the gestational increase of Lpcat1 was blocked in lungs of *Src-1/-2* double-deficient fetuses. Further, PAF or SP-A injection into the AF at 17.5 dpc rescued the parturition delay, enhanced uterine NF-κB activation and *CAP* gene

expression and promoted luteolysis in *Src-1/2*-deficient mice (70). These collective findings further demonstrate the role of the fetal lung in producing signals for the initiation of labor when surfactant production is increased, and that SRC-1/2 coactivators serve crucial roles through enhanced production of SP-A and PAF (**Figure 3**).

P_4/PR MAINTAINS MYOMETRIAL QUIESCENCE VIA CONCERTED MECHANISMS

As described below, P_4/PR maintains myometrial quiescence through a number of cooperative mechanisms. These include: (1) inhibiting transcriptional activity of the pro-inflammatory transcription factors, nuclear factor κB (NF-κB) (110) and activating protein 1 (AP-1) (111), via direct interaction and recruitment of corepressors (10, 111, 112); (2) inducing inhibitors of proinflammatory transcription factor activation (IκBα, MKP-1) (110, 113–115); (3) upregulating expression of transcriptional repressors of *CAP* genes (e.g., ZEB1) (56, 116) (**Figure 4**).

P_4/PR Has Anti-inflammatory Actions in the Myometrium

We (110, 114, 117) and others (46, 118–120) have obtained compelling evidence that P_4/PR maintains myometrial quiescence through its action to block inflammation (**Figure 4**). Using hTERT-HM cells, we observed that P_4/PR serves an anti-inflammatory role by antagonizing the activation of NF-κB and preventing the induction of COX-2 (117, 121), proinflammatory cytokines (10) and *CAP* genes (56). Using chromatin immunoprecipitation (ChIP), we found that P_4 treatment of the hTERT-HM cells prevented interleukin-1 (IL-1)-induced binding of endogenous NF-κB p65 to NF-κB response elements in the *COX-2* and *IL-8* promoters (10, 110). The P_4-mediated inhibition of proinflammatory and *CAP* gene transcription may be caused, in part, by the direct binding of PR to p65 (122) to inhibit NF-κB DNA-binding and transcriptional activity.

P_4/PR can also block NF-κB activation and inflammation by increasing the expression of IκBα, which sequesters NF-κB in the cytoplasm and prevents its activation (110) (**Figure 4**). P_4 treatment of hTERT-HM cells rapidly induced expression of IκBα, which preceded the effect of P_4 to inhibit IL-1β-induced COX-2 expression (110). Moreover, P_4 blocked the IL-1β-mediated decrease in IκBα protein, which suggested P_4/PR inhibition of the proteasome pathway (123). Consequently, more NF-κB remains sequestered in the cytoplasm in an inactive state. Progesterone inhibition of NF-κB activation by upregulation of IκBα has also been reported in Mϕ (124) and breast cancer (113, 125) cell lines. The anti-inflammatory actions of P_4/PR were further mediated by the upregulation of the mitogen-activated protein kinase (MAPK) inhibitor, MAPK phosphatase-1/dual specificity phosphatase 1 (MKP-1/DUSP1) (114, 115). Inhibition of MAPK, in turn, inhibits activation of NF-κB and AP-1 (126–128).

PR Inhibits Proinflammatory and *CAP* Gene Expression by Recruitment of Corepressors

To define the mechanisms whereby P_4 inhibits proinflammatory and *CAP* gene expression in the myometrium, we analyzed the capacity of PR_{WT} *vs.* various PR mutants to mediate P_4 inhibition of proinflammatory gene expression in hTERT-HM human myometrial cells. These included a sumoylation mutant, a hinge domain mutation to prevent PR dimerization, and mutations of three amino acids in the DNA-binding domain (DBD). We observed that mutation of the PR-DBD had the most profound effect to prevent P_4-inhibition of proinflammatory genes (10). Consequently, P_4-mediated transrepression was significantly reduced in cells stably expressing a PR-A or PR-B DNA-binding domain mutant (PR_{mDBD}), compared to cells expressing PR_{WT}. ChIP analysis of the hTERT-HM cells revealed that P_4/PR_{WT} transrepressive activity was associated with P_4-induction of PR recruitment and inhibition of NF-κB p65 and RNA Pol II recruitment to an NF-κB response element in the *COX-2* and *IL-8* promoters. Importantly, in response to P_4 treatment, equivalent recruitment of PR_{WT} and PR_{mDBD} to *COX-2* and *IL-8* promoters was observed. This suggested that the inhibitory effects of P_4/PR on COX-2 and IL-8 expression were not mediated by direct DNA binding, but most likely by tethering to NF-κB. This led us to postulate that nuclear proteins interacting with the PR-DBD may mediate transrepression by P_4/PR. Using immunoprecipitation, followed by mass spectrometry, we identified proteins that interacted strongly with PR_{WT} and weakly with the PR_{DBD} mutants. Among these was the transcriptional repressor, GATA Zinc Finger Domain Containing 2B (GATAD2B), which interacted with the PR-DBD and was required for P_4/PR suppression of *proinflammatory* and *CAP* gene expression (10). Accordingly, P_4 treatment of PR_{WT} hTERT-HM cells increased recruitment of endogenous GATAD2B to *COX-2* and *IL-8* promoters, whereas, siRNA knockdown of endogenous GATAD2B significantly reduced P_4/PR_{WT} transrepression of *COX-2* and *IL-8*. Notably, GATAD2B expression decreased significantly in pregnant mouse and human myometrium during labor (10). Together, our findings suggest that GATAD2B serves as a novel mediator of P_4/PR suppression of proinflammatory and *CAP* genes during pregnancy. Thus, the decline in GATAD2B expression near term may contribute to the loss of PR function leading to labor.

The induction of *CX43* expression by proinflammatory stimuli in the pregnant myometrium is mediated, in part, by increased transcriptional activity of members of the AP-1 family, which comprises Fos/Jun heterodimers or Jun/Jun homodimers. Fos/Jun heterodimers were found to be strong inducers of *CX43* expression compared to Jun/Jun homodimers, which were relatively weak (129). P_4 acting through PR-B was found to repress *CX43* expression by recruiting inactive Jun/Jun homodimers and the $P54^{nrb}$/Sin3A/HDAC corepressor complex to the *CX43* promoter (130). Near term, it was suggested that the increased metabolism of P_4 by 20α-hydroxysteroid dehydrogenase (20α-HSD) in myometrium (131) and an inflammation-induced increase in the PR-A/PR-B ratio (8, 132,

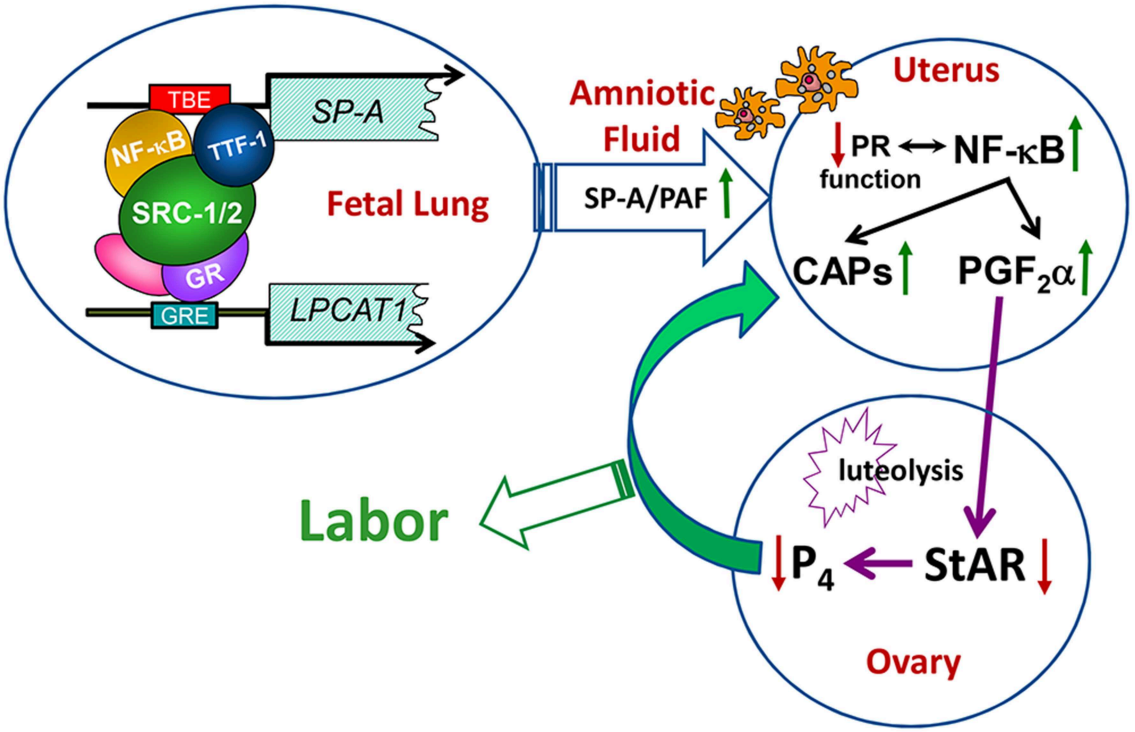

FIGURE 3 | Transcriptional upregulation of the genes encoding SP-A and LPCAT1 in the fetal lung initiates a signaling cascade from fetus to mother that contributes to the initiation of parturition. Toward term, there is increased activation and binding of key transcription factors (e.g., thyroid transcription factor 1 and NF-κB) to response elements upstream of the *SP*-A gene, and of the glucocorticoid receptor (GR) to response elements upstream of the *LPCAT1* gene. These bound transcription factors recruit SRC-1 and SRC-2 to the promoters of these genes, resulting in increased *SP-A* and *LPCAT1* expression and the secretion of SP-A and PAF into amniotic fluid. These immune modulators activate immune cells that migrate into fetal membranes, decidua, and myometrium to increase production of proinflammatory cytokines leading to an activation of inflammatory transcription factors (e.g., NF-κB and AP-1). This contributes to the decline in PR function, with increased contractile (*CAP*) gene expression and synthesis of $PGF_2\alpha$, which circulates to the ovary to inhibit steroidogenic acute regulatory protein (StAR), promote luteolysis and cause a decline in circulating P_4. The decrease in P_4/PR function causes further upregulation of *CAP* gene expression and culminates in labor. Reproduced from Gao et al. (70).

133) caused PR-A to become free of ligands. The unliganded PR is then proposed to recruit relatively active Fra2/JunD heterodimers (129, 130), resulting in the activation of *CX43* expression. This switch may allow transformation of PR-A to an activator of *CX43* expression.

P_4/PR Maintains Myometrial Quiescence via Induction of ZEB1/2 and STAT5b

ZEB1 and ZEB2

Our findings reveal that P_4/PR maintains myometrial quiescence, in part, by the induction of the zinc finger E-box-binding transcriptional repressor, ZEB1/TCF8/δEF1 (**Figure 4**), which binds to promoters of the *CAP* genes, *OXTR,* and *CX43* and the gene encoding members of the miR-200 family and represses their expression. Downregulation of miR-200s promotes further upregulation of ZEB1 and the related transcription factor, ZEB2/SIP2 (56) (**Figure 2**). Zeb1 was found to be expressed highly in mouse myometrium (116) and to be upregulated by P_4/PR (134). We observed that the expression of Zeb1 and Zeb2 was elevated in myometrial tissues of 15.5 dpc pregnant mice and decreased precipitously toward term with the decline in circulating P_4 and in PR function (56). Conversely, treatment of pregnant mice with RU486 or with the bacterial endotoxin, lipopolysaccharide (LPS), to induce preterm labor (56) caused significant inhibition of Zeb1/2 expression. ZEB1 and ZEB2 levels also were decreased in term myometrial tissues from women in labor, *vs.* tissues from women not in labor at term (56). ChIP-qPCR analysis of pregnant mouse myometrium revealed that endogenous Zeb1 bound to E-box-containing regions of the mouse *Cx43* and *Oxtr* promoters at relatively high levels at 15.5 dpc and declined markedly at term (56). Importantly, ZEB1 overexpression in human myometrial hTERT-HM cells caused a pronounced inhibition of OXTR and CX43 mRNA levels and blocked oxytocin-induced contraction of these cells using an *in vitro* contraction assay (56). Thus, the decrease in Zeb1 expression and binding to *CAP* gene promoters toward term likely promoted upregulation of *OXTR* and *CX43* leading to parturition.

To further assess the effects of P_4 on Zeb expression, timed-pregnant mice were injected with P_4 or vehicle daily between 15.5 and 18.5 dpc. P_4 treatment, which delayed labor, caused a significant upregulation of Zeb1 and inhibited myometrial *CX43* and *OXTR* gene expression, compared to vehicle injected mice. A similar inductive effect of P_4 on ZEB1 expression was observed in cultured T47D breast cancer cells. The stimulatory effect of P_4 on

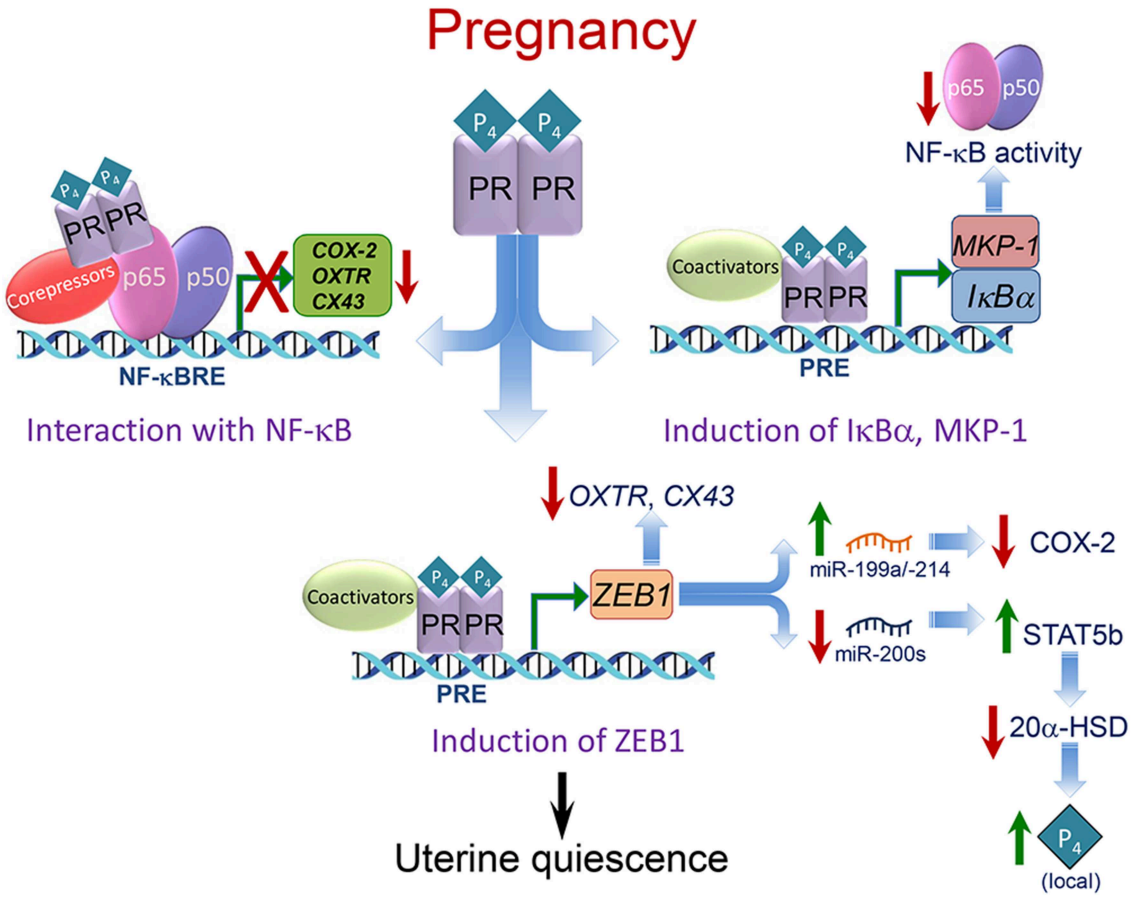

FIGURE 4 | Mechanisms whereby P_4/PR maintains myometrial quiescence during pregnancy. Multiple mechanisms mediate effects of P_4/PR to suppress proinflammatory and *CAP* gene expression in the myometrium. As shown in the upper left, P_4/PR exerts anti-inflammatory effects by tethering to NF-κB p65 bound to NF-κB response elements (NF-κBRE) within the promoters of proinflammatory and *CAP* genes. This stimulates the recruitment of corepressors, such as GATAD2B. Similar mechanisms are believed to occur at AP-1 sites upstream of *CAP* gene promoters, whereby PR interacts with FOS/JUN and recruits corepressors, such as $P54^{nrb}$/Sin3A/HDAC. As shown in the upper right, P_4/PR inhibits NF-κB activation and proinflammatory and *CAP* gene expression in myometrium by binding to the *IκBα* promoter and enhancing IκBα expression. The increased cytoplasmic levels of IκBα interact with NF-κB to block its activation and translocation to the nucleus. P_4/PR also exerts anti-inflammatory effects in myometrium by binding to the *MKP1/DUSP1* promoter to increase its expression and prevent MAPK activation, resulting in the inhibition of NF-κB and AP-1 activity. As shown in the lower panel, P_4/PR blocks *CAP* gene expression by binding to the *ZEB1* promoter and enhancing its expression. The increased levels of ZEB1 bind to the promoters of *CAP* genes to inhibit their expression, activates expression of the *miR-199a/-214* cluster to inhibit expression of their target, COX-2, and inhibits miR-200 expression, leading to further induction of ZEB1 and ZEB2 as well as STAT5b, causing suppression of 20α-HSD and increased local P_4 accumulation. Modified from Renthal et al. (58).

ZEB1 expression was mediated at the level of gene transcription by the direct binding of PR to response element(s) within the ZEB1 promoter (56). Notably, ZEB2 is not directly regulated by P_4/PR (56).

ZEB1 and ZEB2 are directly targeted by all five members of the highly conserved miR-200 family (135–138), which significantly increase in mouse and human myometrium toward term and in labor, in association with the decline in ZEB1 and ZEB2 expression (56). ZEB1 and ZEB2 also negatively regulate miR-200 expression. Accordingly, ZEB1/2 and miR-200s exist in a hormonally-regulated negative feedback loop (135–138). Thus, during pregnancy, elevated P_4/PR increases the expression of ZEB1, which suppresses the miR-200 family, as well as contraction-associated genes. The decrease in miR-200 expression further upregulates ZEB1 and increases the expression of ZEB2. Near term, the decline in circulating P_4 and/or PR function causes the downregulation of ZEB1 expression, and consequent upregulation of the miR-200 family, further suppressing ZEB1 and ZEB2. This de-represses *CAP* gene expression, resulting in increased uterine contractility and labor (**Figure 2**).

In parallel with our discovery of the gestational upregulation of the miR-200 family, we found that the conserved *miR-199a/-214 cluster* was significantly downregulated in mouse myometrium near term (56, 57), and in myometrial biopsies from women in-labor, compared to those not-in-labor at

term (57). The *miR-199a/-214* cluster, comprised of miR-199a-5p, miR-199a-3p, and miR-214, are encoded within a 6-kb anti-sense transcript of the *Dynamin3* gene (*Dnm3os*), which is highly expressed in pregnant uterus (139). We observed that E_2 treatment of ovariectomized mice suppressed, whereas P_4 enhanced, uterine miR-199a-3p/-214 expression (57). Interestingly, these opposing hormonal effects were mediated by ZEB1, which, as described above, is induced by P_4 (56, 116) and inhibited by E_2 (57). ZEB1 binds directly to *the miR199a/-214* promoter to activate its transcription (57) (**Figure 2**). Importantly, miR-199a-3p and miR-214 both target the mRNA for COX-2 (57, 140). Thus, miR-199a-3p and miR-214 maintain uterine quiescence by suppressing the synthesis of contractile prostaglandins. These collective findings revealed the intriguing central role of ZEB1 as an inhibitor of the *miR-200* family and an inducer of the *miR-199a/-214 cluster*, which are oppositely regulated by P_4 and E_2 (**Figure 2**).

Signal Transducer and Activator of Transcription (STAT)5b

Increased local metabolism of P_4 to inactive products near term in the uterus and cervix has been suggested to contribute to the decline in PR function that is crucial for the initiation of parturition in all mammals (19, 141–144). The finding that mice with a deletion of the gene encoding the P_4-metabolizing enzyme, 20α-hydroxysteroid dehydrogenase (20α-HSD), manifested a significant delay in the timing of parturition, indicates its important role (20). In myometrium of pregnant women at term, a pronounced decrease in the ratio of P_4 to 20α-dihydroprogesterone (20α-OHP), an inactive metabolite of P_4 generated by 20α-HSD, were observed (144). In the course of our studies, we discovered that miR-200s directly target the P_4-induced transcription factor STAT5b (131), which is a negative regulator of 20α-HSD in reproductive tissues (20, 145). Thus, the upregulation of miR-200 expression in mouse myometrium near term and in human myometrium during labor (56) was associated with suppression of STAT5b and induction of 20α-HSD (131). In contrast, throughout most of pregnancy, increased P_4 levels upregulate ZEB1 and inhibit miR-200 expression in the myometrium (56). This, in turn, allows upregulation of STAT5b, which inhibits 20α-HSD expression (131) to maintain elevated endogenous levels of P_4 and myometrial quiescence (**Figure 2**).

P_4/PR Induction of Caspases in the Pregnant Myometrium During Mid-Gestation Maintains Quiescence

During early to mid-pregnancy in rats there is a high rate of myometrial cell proliferation and hyperplasia, exemplified by increased BrdU incorporation and PCNA staining in the longitudinal smooth muscle layer (146). This declines precipitously by 17 dpc (term = 23 dpc) and is accompanied by an increase in cellular hypertrophy. From 12 to 15 dpc, there was a remarkable induction of the stress-induced caspase cascade (cleaved caspases 9, 3, 6, and 7) (146). However, this was not accompanied by evidence for apoptosis. Rather, it was suggested that caspase activation may cause inhibition of myometrial proliferative activity and promote the transition to hypertrophy and smooth muscle cell differentiation (146). Similar inductive changes in activation of the caspase cascade were observed in pregnant mouse myometrium from 12 to 15 dpc (term = 19 dpc) (147). Importantly, the activation of caspase 3 was found to be stimulated by P_4 treatment. Moreover, caspase 3 activation was accompanied by the cleavage of myocyte contractile proteins, smooth muscle α- and γ-actins (147) as well as downregulation of the gap junction protein Cx43 (148). Thus, an important mechanism whereby P_4/PR maintains myometrial quiescence during pregnancy is via its action to induce the active caspase cascade and cause degradation of proteins involved in myometrial contractility. The decline in PR function in the pregnant myometrium toward term results in decreased caspase activation and allows for the increase in contractile protein accumulation. Notably, caspase activation in uterine myocytes was also associated with induction of the endoplasmic reticulum stress response (ERSR), which is likely enhanced by physiological/mechanical stimuli (149). The ERSR was reduced near term by upregulation of the adaptive unfolded protein response (UPR), resulting in a decline in active caspase 3 and for the induction of contractile proteins (149).

MECHANISMS FOR THE DECLINE IN PR FUNCTION LEADING TO PARTURITION

The decline in PR function leading to parturition, which is fundamental and critical for species survival, is mediated by multiple complementary mechanisms, several of which are discussed below. It is likely that all of these processes are regulated by an increased inflammatory response within the myometrium at term, resulting in activation of NF-κB and AP-1 transcription factors. In various cell types, activation of NF-κB represses PR transcriptional activity, while PR activation also represses NF-κB-mediated transcription (28, 122).

Altered PR Isoform Expression and Posttranslational Modification in the Myometrium Toward Term

As mentioned, PR-A, which is truncated at its N-terminus, contains only two of the three transcriptional activation domains that are present in PR-B. Thus, in certain cell- and gene-specific contexts, including human myometrial cells, PR-A has been found to repress PR-B transcriptional activity (5–7). PR-A and PR-B are differentially regulated in the human myometrium during pregnancy (8); the ratio of PR-A to PR-B mRNA (9) and protein (7) was observed to be significantly higher in term myometrium from women in labor compared to those not in labor. Moreover, in hTERT-HM myometrial cells stably expressing PR-A or PR-B, it was observed that P_4 had decreased anti-inflammatory activity in PR-A-expressing cells when compared to those expressing PR-B (10). Furthermore, proinflammatory stimuli specifically increased phosphorylation of the PR-A isoform on Ser-344/345 in a P_4-dependent manner and enhanced its ability to antagonize the anti-inflammatory activity of PR-B (133). Notably, in term myometrium from

women in-labor vs. not-in-labor, phosphorylation of Ser-345 occurred exclusively on PR-A, and the abundance of phospho-Ser-345-PR-A relative to total PR-A was increased significantly in laboring vs. non-laboring myometrium in association with increased NF-κB activation (133).

A third PR isoform, PR-C (∼60 kDa), which is truncated from the N-terminus and lacks part of the DNA binding domain, is primarily cytoplasmic in its localization (150, 151). Since PR-C can bind P_4 (152), it may inhibit PR function by sequestering P_4 and/or by physically interacting with PR-B to reduce its DNA-binding capacity (150). In fundal myometrium from women before and after the initiation of labor at term, we observed a labor-associated increase in PR-C mRNA and a ∼60 kDa immunoreactive PR protein. This was temporally and spatially associated with activation of NF-κB (27). A temporal increase in a truncated 60 kDa PR isoform was also observed in mouse uterus near term. Notably, the identity of truncated PR isoforms in the pregnant uterus (8, 153) requires further study.

Decreased Expression of Selected Coregulators in the Myometrium Toward Term

P_4/PR activation of target gene expression is dependent upon recruitment of coactivator complexes, which contain histone acetyltransferases and cause an opening of chromatin structure (154, 155). Previously, we observed that expression of selected steroid receptor coactivators (SRC) and the histone acetyltransferase, CREB-binding protein (CBP), declined in myometrium of women in labor, compared to non-laboring myometrium, and were profoundly decreased in myometrial tissues of pregnant mice at term (156). This was associated with decreased levels of acetylated histone H3. Remarkably, treatment of pregnant mice with the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), during late gestation increased myometrial histone acetylation and delayed parturition by 24–48 h (156). Subsequently, it was reported that HDAC inhibitors suppressed proinflammatory gene expression in cultured human myometrial cells (157) and strongly inhibited contractility of human myometrial strips (158). Collectively, these findings suggest that decreased expression of PR coactivators and in histone acetylation in the myometrium during late gestation may impair the capacity of P_4/PR to upregulate genes that maintain myometrial quiescence and increase sensitivity of the uterus to prostaglandins and other contractile factors. In cultured human myometrial cells, the effect of TNFα to inhibit SRC-1 and SRC-2 expression mediated TNFα inhibition of PR-B transcriptional activity (159). Thus, the decline in coactivators in the myometrium near term may be caused by induction of proinflammatory mediators. Moreover, corepressors, GATAD2B (10) and p54nrb (non-POU-domain-containing, octamer binding protein) (111), which interact with PR and mediate its capacity to repress proinflammatory and *CAP* gene expression, were found to decrease in rodent myometrium at term, further contributing to the decline in PR function.

Increased Metabolism of P_4 Toward Term Contributes to the Decline in PR Function Leading to Parturition

As mentioned, in women, circulating P_4 levels remain elevated throughout pregnancy and labor due to the maintenance of placental P_4 production (160). Furthermore, levels of PR remain elevated in reproductive tissues during pregnancy and into labor (12). Even in rodents, where P_4 production by the corpus luteum declines precipitously near term, the levels of P_4 in circulation remain higher than the K_d for binding to PR (17). As mentioned, in the myometrium of pregnant women at term, there is a pronounced decrease in the ratio of P_4 to 20α-dihydroprogesterone (144), an inactive P_4 metabolite generated by the enzyme 20α-HSD. In mice, the initiation of parturition is accompanied by increased expression of the P_4-metabolizing enzymes, 20α-HSD in the uterus (131) and 5α-reductase type I in the cervix (18, 19). Accordingly, gene-targeted mice lacking 5α-reductase type I fail to deliver because of impaired cervical ripening, even though maternal circulating P_4 levels decline normally (18, 19). Similarly, *20α-HSD* knockout mice manifested severely delayed parturition (20, 161). Thus, increased local metabolism of P_4 in the uterus and cervix near term contributes to the decline in PR function and is crucial for the initiation of parturition (19, 141–144).

CONCLUSIONS

Throughout pregnancy, the critical role of P_4/PR in maintaining myometrial quiescence is principally mediated by its capacity to inhibit inflammatory pathways and to suppress *CAP* gene expression. As depicted in **Figure 4**, this occurs via several cooperative mechanisms that include: tethering of PR to the inflammatory transcription factors, NF-κB or AP-1, with recruitment of corepressors (10, 110–112, 130); PR promoter binding and transcriptional activation of genes encoding the NF-κB suppressor, IκBα (110), and the MAPK inhibitor, MKP1 (114). Increased P_4/PR also upregulates expression of transcription factor ZEB1, which interacts directly with the promoters of the *OXTR* and *CX43* genes to inhibit their expression and suppresses expression of the miR-200 family (56). Decreased miR-200 expression allows upregulation of its target, STAT5b, a transcriptional inhibitor of *20α-HSD*, so that P_4 metabolism in the myometrium is prevented (131). The increased ZEB1 also upregulates the expression of members of the miR-199a/-214 cluster, which directly target COX-2, resulting in the suppression of contractile prostaglandin synthesis (57) (**Figures 2**, **4**). P_4 also contributes to myometrial quiescence by activation of the caspase cascade, which maintains low levels of myocyte contractile proteins through increased caspase-mediated degradation (147).

The transition of the pregnant myometrium to an inflammatory, contractile state at term is affected by cooperative signals from the mother and fetus (**Figure 1**). Our findings suggest that appropriate timing for parturition is mediated, in part, by the induction of surfactant signals, SP-A and PAF, produced by the fetal lung and secreted into amniotic fluid where they interact with fetal Mϕ to alter their phenotypic

state (23, 69, 70). These activated immune cells then migrate to the maternal uterus (23) where they promote an inflammatory response with activation of NF-κB and AP-1, leading to a decline in PR function and increased expression of proinflammatory and *CAP* genes in the myometrium (**Figures 1**, **3**). The decline in PR function in human myometrium is thought to be mediated by: an inflammation-induced increase in PR-A, relative to PR-B isoform expression (133), with possible upregulation of other truncated PR isoforms (27); a decreased expression of ZEB1 and coordinate induction of miR-200 family expression (56), resulting in suppression of STAT5b, upregulation of 20α-HSD and with increased local metabolism of P_4 (131); the direct interaction of NF-κB p65 with PR (122); a decline in PR coactivators (156). The decline in ZEB1 also contributes to upregulation of *OXTR* and *CX43* (56) and a decrease in *miR-199a/-214* expression with the induction of their target, COX-2 (57), and of $PGF_2\alpha$ synthesis (**Figure 1**). Toward term, the increase in E_2 production and ERα activation in the myometrium results in increased proinflammatory and *CAP* gene expression, which is mediated, in part, by inhibition of ZEB1/2 (57) and by the decreased expression of miR-181b, allowing for the upregulation of its targets, ERα, TNFα and c-FOS (53). These highly coordinated molecular events, together with increased myometrial stretch and a decline in PR corepressor expression culminate in the increased myometrial contractility leading to parturition.

Our extensive knowledge of the mechanisms that underlie myometrial quiescence during pregnancy and its transition to a contractile state prior to parturition has led to the identification of a number of conserved potential therapeutic targets for the prevention of preterm labor and its consequences. Several of these targets include miRNA clusters and families that are coordinately upregulated or downregulated toward term and target a number of signaling molecules and pathways. Of considerable importance is the miR-200 family, which is markedly upregulated in pregnant human and mouse myometrium toward term. miR-200 family members target and downregulate expression of ZEB1 and ZEB2, leading to increased contractile gene expression and the suppression of STAT5b, which results in increased local metabolism of P_4 by 20α-HSD (131). The decline in ZEB1/2 also causes decreased miR-199a and miR-214 expression, which both independently target the contractile gene, *PTGS2/COX2*, resulting in an increase in prostaglandin synthesis (57). The induction of miR-200s also directly targets PR (162), which may contribute to the loss of its function. Thus, anti-miR-200 therapy could form the basis for a comprehensive, multifactorial and highly effective therapeutic strategy for prevention of preterm birth.

AUTHOR CONTRIBUTIONS

CM wrote the manuscript. LG and AM critiqued and edited the manuscript.

FUNDING

The research conducted by these authors reviewed in this article was supported, in part, by NIH P01HD087150 (CM), NIH R01-HL050022 (CM), Prematurity Research Grant #21-FY14-146 from the March of Dimes Foundation (CM) and the National Natural Science Foundation of China (NSFC to LG) 81622020 and 81771608.

REFERENCES

1. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, et al. Born too soon: the global epidemiology of 15 million preterm births. *Reprod Health.* (2013) 10(Suppl 1):S2. doi: 10.1186/1742-4755-10-S1-S2
2. Peltier MR, Drobek CO, Bhat G, Saade G, Fortunato SJ, Menon R. Amniotic fluid and maternal race influence responsiveness of fetal membranes to bacteria. *J Reprod Immunol.* (2012) 96:68–78. doi: 10.1016/j.jri.2012.07.006
3. Kastner P, Bocquel MT, Turcotte B, Garnier JM, Horwitz KB, Chambon P, et al. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. *J Biol Chem*. (1990) 265:12163–7.
4. Conneely OM, Maxwell BL, Toft DO, Schrader WT, O'Malley BW. The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA. *Biochem Biophys Res Commun*. (1987) 149:493–501. doi: 10.1016/0006-291X(87)90395-0
5. Giangrande PH, Kimbrel EA, Edwards DP, McDonnell DP. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol Cell Biol*. (2000) 20:3102–15. doi: 10.1128/MCB.20.9.3102-3115.2000
6. Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol*. (1993) 7:1244–55. doi: 10.1210/mend.7.10.8264658
7. Pieber D, Allport VC, Hills F, Johnson M, Bennett PR. Interactions between progesterone receptor isoforms in myometrial cells in human labour. *Mol Hum Reprod*. (2001) 7:875–9. doi: 10.1093/molehr/7.9.875
8. Merlino AA, Welsh TN, Tan H, Yi LJ, Cannon V, Mercer BM, et al. Nuclear progesterone receptors in the human pregnancy myometrium: evidence that parturition involves functional progesterone withdrawal mediated by increased expression of progesterone receptor-A. *J Clin Endocrinol Metab*. (2007) 92:1927–33. doi: 10.1210/jc.2007-0077
9. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. *J Clin Endocrinol Metab*. (2002) 87:2924–30. doi: 10.1210/jcem.87.6.8609
10. Chen CC, Montalbano AP, Hussain I, Lee WR, Mendelson CR. The transcriptional repressor GATAD2B mediates progesterone receptor suppression of myometrial contractile gene expression. *J Biol Chem*. (2017) 292:12560–76. doi: 10.1074/jbc.M117.791350
11. Virgo BB, Bellward GD. Serum progesterone levels in the pregnant and postpartum laboratory mouse. *Endocrinology*. (1974) 95:1486–90. doi: 10.1210/endo-95-5-1486
12. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev*. (2000) 21:514–50. doi: 10.1210/er.21.5.514
13. Frydman R, Lelaidier C, Baton-Saint-Mleux C, Fernandez H, Vial M, Bourget P. Labor induction in women at term with mifepristone (RU 486): a double-blind, randomized, placebo-controlled study. *Obstet Gynecol*. (1992) 80:972–5. doi: 10.1016/0020-7292(93)90660-O

14. Elliott CL, Brennand JE, Calder AA. The effects of mifepristone on cervical ripening and labor induction in primigravidae. *Obstet Gynecol*. (1998) 92:804–9. doi: 10.1097/00006250-199811000-00013
15. Stenlund PM, Ekman G, Aedo AR, Bygdeman M. Induction of labor with mifepristone–a randomized, double-blind study versus placebo. *Acta Obstet Gynecol Scand*. (1999) 78:793–8. doi: 10.1080/j.1600-0412.1999.780910.x
16. Chwalisz K. The use of progesterone antagonists for cervical ripening and as an adjunct to labour and delivery. *Hum Reprod*. (1994) 9(Suppl 1):131–61. doi: 10.1093/humrep/9.suppl_1.131
17. Pointis G, Rao B, Latreille MT, Mignot TM, Cedard L. Progesterone levels in the circulating blood of the ovarian and uterine veins during gestation in the mouse. *Biol Reprod*. (1981) 24:801–5. doi: 10.1095/biolreprod24.4.801
18. Mahendroo MS, Cala KM, Russell DW. 5α-reduced androgens play a key role in murine parturition. *Mol Endocrinol*. (1996) 10:380–92. doi: 10.1210/mend.10.4.8721983
19. Mahendroo MS, Porter A, Russell DW, Word RA. The parturition defect in steroid 5α-reductase type 1 knockout mice is due to impaired cervical ripening. *Mol Endocrinol*. (1999) 13:981–92. doi: 10.1210/mend.13.6.0307
20. Piekorz RP, Gingras S, Hoffmeyer A, Ihle JN, Weinstein Y. Regulation of progesterone levels during pregnancy and parturition by signal transducer and activator of transcription 5 and 20alpha-hydroxysteroid dehydrogenase. *Mol Endocrinol*. (2005) 19:431–40. doi: 10.1210/me.2004-0302
21. Cox SM, Casey ML, MacDonald PC. Accumulation of interleukin-1beta and interleukin-6 in amniotic fluid: a sequela of labour at term and preterm. *Hum Reprod Update*. (1997) 3:517–27. doi: 10.1093/humupd/3.5.517
22. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, et al. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Hum Reprod*. (1999) 14:229–36. doi: 10.1093/humrep/15.1.229
23. Condon JC, Jeyasuria P, Faust JM, Mendelson CR. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. *Proc Natl Acad Sci USA*. (2004) 101:4978–83. doi: 10.1073/pnas.0401124101
24. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol Hum Reprod*. (2003) 9:41–5. doi: 10.1093/molehr/gag001
25. Mendelson CR, Montalbano AP, Gao L. Fetal-to-maternal signaling in the timing of birth. *J Steroid Biochem Mol Biol*. (2017) 170:19–27. doi: 10.1016/j.jsbmb.2016.09.006
26. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. *Semin Reprod Med*. (2007) 25:21–39. doi: 10.1055/s-2006-956773
27. Condon JC, Hardy DB, Kovaric K, Mendelson CR. Upregulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of NF-kappaB may contribute to the onset of labor through inhibition of PR function. *Mol Endocrinol*. (2006) 20:764–75. doi: 10.1210/me.2005-0242
28. Allport VC, Pieber D, Slater DM, Newton R, White JO, Bennett PR. Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. *Mol Hum Reprod*. (2001) 7:581–6. doi: 10.1093/molehr/7.6.581
29. Lee YS, Terzidou V, Lindstrom T, Johnson M, Bennett PR. The role of CCAAT/enhancer-binding protein beta in the transcriptional regulation of COX-2 in human amnion. *Mol Hum Reprod*. (2005) 11:853–8. doi: 10.1093/molehr/gah194
30. Elliott CL, Allport VC, Loudon JA, Wu GD, Bennett PR. Nuclear factor-κB is essential for up-regulation of interleukin-8 expression in human amnion and cervical epithelial cells. *Mol Hum Reprod*. (2001) 7:787–90. doi: 10.1093/molehr/7.8.787
31. Olson DM. The role of prostaglandins in the initiation of parturition. *Best Pract Res Clin Obstet Gynaecol*. (2003) 17:717–30. doi: 10.1016/S1521-6934(03)00069-5
32. Chow L, Lye SJ. Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. *Am J Obstet Gynecol*. (1994) 170:788–95. doi: 10.1016/S0002-9378(94)70284-5
33. Fuchs AR, Fuchs F, Husslein P, Soloff MS. Oxytocin receptors in the human uterus during pregnancy and parturition. *Am J Obstet Gynecol*. (1984) 150:734–41. doi: 10.1016/0002-9378(84)90677-X
34. Soloff MS, Cook DL Jr, Jeng YJ, Anderson GD. *In situ* analysis of interleukin-1-induced transcription of *COX-2* and *IL-8* in cultured human myometrial cells. *Endocrinology*. (2004) 145:1248–54. doi: 10.1210/en.2003-1310
35. Rauk PN, Chiao JP. Interleukin-1 stimulates human uterine prostaglandin production through induction of cyclooxygenase-2 expression. *Am J Reprod Immunol*. (2000) 43:152–9. doi: 10.1111/j.8755-8920.2000.430304.x
36. Shynlova O, Tsui P, Dorogin A, Lye SJ. Monocyte chemoattractant protein-1 (CCL-2) integrates mechanical and endocrine signals that mediate term and preterm labor. *J Immunol*. (2008) 181:1470–9. doi: 10.4049/jimmunol.181.2.1470
37. Sooranna SR, Lee Y, Kim LU, Mohan AR, Bennett PR, Johnson MR. Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. *Mol Hum Reprod*. (2004) 10:109–13. doi: 10.1093/molehr/gah021
38. Goldenberg RL, Iams JD, Miodovnik M, Van Dorsten JP, Thurnau G, Bottoms S, et al. The preterm prediction study: risk factors in twin gestations. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *Am J Obstet Gynecol*. (1996) 175:1047–53. doi: 10.1016/S0002-9378(96)80051-2
39. Esplin MS, Peltier MR, Hamblin S, Smith S, Fausett MB, Dildy GA, et al. Monocyte chemotactic protein-1 expression is increased in human gestational tissues during term and preterm labor. *Placenta*. (2005) 26:661–71. doi: 10.1016/j.placenta.2004.09.012
40. Adams Waldorf KM, Singh N, Mohan AR, Young RC, Ngo L, Das A, et al. Uterine overdistention induces preterm labor mediated by inflammation: observations in pregnant women and nonhuman primates. *Am J Obstet Gynecol*. (2015) 213:830. doi: 10.1016/j.ajog.2015.08.028
41. Hua R, Pease JE, Sooranna SR, Viney JM, Nelson SM, Myatt L, et al. Stretch and inflammatory cytokines drive myometrial chemokine expression via NF-kappaB activation. *Endocrinology*. (2012) 153:481–91. doi: 10.1210/en.2011-1506
42. Kelly RW, Carr GG, Riley SC. The inhibition of synthesis of a beta-chemokine, monocyte chemotactic protein-1 (MCP-1) by progesterone. *Biochem Biophys Res Commun*. (1997) 239:557–61. doi: 10.1006/bbrc.1997.7502
43. Challis JR. Sharp increase in free circulating oestrogens immediately before parturition in sheep. *Nature*. (1971) 229:208. doi: 10.1038/229208a0
44. Buster JE, Chang RJ, Preston DL, Elashoff RM, Cousins LM, Abraham GE, et al. Interrelationships of circulating maternal steroid concentrations in third trimester pregnancies. II. C_{18} and C_{19} steroids: estradiol, estriol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, Δ^5-androstenediol, Δ^4-androstenedione, testosterone, and dihydrotestosterone. *J Clin Endocrinol Metab*. (1979) 48:139–42. doi: 10.1210/jcem-48-1-139
45. Wu WX, Myers DA, Nathanielsz PW. Changes in estrogen receptor messenger ribonucleic acid in sheep fetal and maternal tissues during late gestation and labor. *Am J Obstet Gynecol*. (1995) 172:844–50. doi: 10.1016/0002-9378(95)90009-8
46. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. *Biol Reprod*. (1999) 60:1158–65. doi: 10.1095/biolreprod60.5.1158
47. Murata T, Narita K, Honda K, Matsukawa S, Higuchi T. Differential regulation of estrogen receptor alpha and beta mRNAs in the rat uterus during pregnancy and labor: possible involvement of estrogen receptors in oxytocin receptor regulation. *Endocr J*. (2003) 50:579–87. doi: 10.1507/endocrj.50.579

48. Piersanti M, Lye SJ. Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activator protein-1, c-fos. *Endocrinology*. (1995) 136:3571–8. doi: 10.1210/endo.136.8.7628395
49. Tsuboi K, Sugimoto Y, Iwane A, Yamamoto K, Yamamoto S, Ichikawa A. Uterine expression of prostaglandin H2 synthase in late pregnancy and during parturition in prostaglandin F receptor-deficient mice. *Endocrinology*. (2000) 141:315–24. doi: 10.1210/en.141.1.315
50. Engstrom T. The regulation by ovarian steroids of prostaglandin synthesis and prostaglandin-induced contractility in non-pregnant rat myometrium. Modulating effects of isoproterenol. *J Endocrinol*. (2001) 169:33–41. doi: 10.1677/joe.0.1690033
51. Gibb W. The role of prostaglandins in human parturition. *Ann Med*. (1998) 30:235–41. doi: 10.3109/07853899809005850
52. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol*. (2000) 74:311–7. doi: 10.1016/S0960-0760(00)00108-4
53. Gao L, Wang G, Liu W, Kinser H, Franco HL, Mendelson CR. Reciprocal feedback between miR-181a and E_2/ERα in myometrium enhances inflammation leading to labor. *J Clin Endocrinol Metab*. (2016) 101:3646–56. doi: 10.1210/jc.2016-2078
54. Mitchell JA, Lye SJ. Differential expression of activator protein-1 transcription factors in pregnant rat myometrium. *Biol Reprod*. (2002) 67:240–6. doi: 10.1095/biolreprod67.1.240
55. Wu C, Gong Y, Yuan J, Zhang W, Zhao G, Li H, et al. microRNA-181a represses ox-LDL-stimulated inflammatory response in dendritic cell by targeting c-Fos. *J Lipid Res*. (2012) 53:2355–63. doi: 10.1194/jlr.M028878
56. Renthal NE, Chen CC, Williams KC, Gerard RD, Prange-Kiel J, Mendelson CR. miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor. *Proc Natl Acad Sci USA*. (2010) 107:20828–33. doi: 10.1073/pnas.1008301107
57. Williams KC, Renthal NE, Gerard RD, Mendelson CR. The microRNA (miR)-199a/214 cluster mediates opposing effects of progesterone and estrogen on uterine contractility during pregnancy and labor. *Mol Endocrinol*. (2012) 26:1857–67. doi: 10.1210/me.2012-1199
58. Renthal NE, Williams KC, Montalbano AP, Chen CC, Gao L, Mendelson CR. Molecular regulation of parturition: a myometrial perspective. *Cold Spring Harb Perspect Med*. (2015) 5:181–96. doi: 10.1101/cshperspect.a023069
59. Liggins GC, Fairclough RJ, Grieves SA, Kendall JZ, Knox BS. The mechanism of initiation of parturition in the ewe. *Recent Prog Horm Res*. (1973) 29:111–59. doi: 10.1016/B978-0-12-571129-6.50007-5
60. Liggins GC. Premature delivery of foetal lambs infused with glucocorticoids. *J Endocrinol*. (1969) 45:515–23. doi: 10.1677/joe.0.0450515
61. Rainey WE, Rehman KS, Carr BR. The human fetal adrenal: making adrenal androgens for placental estrogens. *Semin Reprod Med*. (2004) 22:327–36. doi: 10.1055/s-2004-861549
62. Robinson BG, Arbiser JL, Emanuel RL, Majzoub JA. Species-specific placental corticotropin releasing hormone messenger RNA and peptide expression. *Mol Cell Endocrinol*. (1989) 62:337–41. doi: 10.1016/0303-7207(89)90022-1
63. Florio P, Cobellis L, Woodman J, Severi FM, Linton EA, Petraglia F. Levels of maternal plasma corticotropin-releasing factor and urocortin during labor. *J Soc Gynecol Investig*. (2002) 9:233–7. doi: 10.1177/107155760200900409
64. Torricelli M, Giovannelli A, Leucci E, De Falco G, Reis FM, Imperatore A, et al. Labor (term and preterm) is associated with changes in the placental mRNA expression of corticotrophin-releasing factor. *Reprod Sci*. (2007) 14:241–5. doi: 10.1177/1933719107300971
65. Keegan CE, Herman JP, Karolyi IJ, O'Shea KS, Camper SA, Seasholtz AF. Differential expression of corticotropin-releasing hormone in developing mouse embryos and adult brain. *Endocrinology*. (1994) 134:2547–55. doi: 10.1210/en.134.6.2547
66. Muglia LJ, Bae DS, Brown TT, Vogt SK, Alvarez JG, Sunday ME, et al. Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. *Am J Respir Cell Mol Biol*. (1999) 20:181–8. doi: 10.1165/ajrcmb.20.2.3381
67. Lopez BA, Newman GE, Phizackerley PJ, Turnbull AC. Surfactant stimulates prostaglandin E production in human amnion. *Br J Obstet Gynaecol*. (1988) 95:1013–7. doi: 10.1111/j.1471-0528.1988.tb06506.x
68. Frenkel RA, Muguruma K, Johnston JM. The biochemical role of platelet-activating factor in reproduction. *Prog Lipid Res*. (1996) 35:155–68. doi: 10.1016/0163-7827(96)00002-1
69. Montalbano AP, Hawgood S, Mendelson CR. Mice deficient in surfactant protein A (SP-A) and SP-D or in TLR2 manifest delayed parturition and decreased expression of inflammatory and contractile genes. *Endocrinology*. (2013) 154:483–98. doi: 10.1210/en.2012-1797
70. Gao L, Rabbitt EH, Condon JC, Renthal NE, Johnston JM, Mitsche MA, et al. Steroid receptor coactivators 1 and 2 mediate fetal-to-maternal signaling that initiates parturition. *J Clin Invest*. (2015) 125:2808–24. doi: 10.1172/JCI78544
71. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. *N Engl J Med*. (2002) 347:2141–8. doi: 10.1056/NEJMra022387
72. Kuroki Y, Takahashi M, Nishitani C. Pulmonary collectins in innate immunity of the lung. *Cell Microbiol*. (2007) 9:1871–9. doi: 10.1111/j.1462-5822.2007.00953.x
73. Hawgood S, Shiffer K. Structures and properties of the surfactant-associated proteins. *Annu Rev Physiol*. (1991) 53:375–94. doi: 10.1146/annurev.ph.53.030191.002111
74. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol*. (2005) 5:58–68. doi: 10.1038/nri1528
75. Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defense. *Annu Rev Physiol*. (2001) 63:521–54. doi: 10.1146/annurev.physiol.63.1.521
76. Kremlev SG, Phelps DS. Surfactant protein A stimulation of inflammatory cytokine and immunoglobulin production. *Am J Physiol Lung Cell Mol Physiol*. (1994) 267:L712–9. doi: 10.1152/ajplung.1994.267.6.L712
77. Phelps DS. Surfactant regulation of host defense function in the lung: a question of balance. *Pediatr Pathol Mol Med*. (2001) 20:269–92. doi: 10.3109/15513810109168822
78. Mitchell MD, MacDonald PC, Casey ML. Stimulation of prostaglandin E2 synthesis in human amnion cells maintained in monolayer culture by a substance(s) in amniotic fluid. *Prostaglandins Leukot Med*. (1984) 15:399–407. doi: 10.1016/0262-1746(84)90138-0
79. Toyoshima K, Narahara H, Furukawa M, Frenkel RA, Johnston JM. Platelet-activating factor. Role in fetal lung development and relationship to normal and premature labor. *Clin Perinatol*. (1995) 22:263–80. doi: 10.1016/S0095-5108(18)30285-9
80. Mendelson CR, Boggaram V. Hormonal control of the surfactant system in fetal lung. *Annu Rev Physiol*. (1991) 53:415–40. doi: 10.1146/annurev.ph.53.030191.002215
81. Korfhagen TR, Bruno MD, Glasser SW, Ciraolo PJ, Whitsett JA, Lattier DL, et al. Murine pulmonary surfactant SP-A gene: cloning, sequence, and transcriptional activity. *Am J Physiol Lung Cell Mol Physiol*. (1992) 263:L546–54. doi: 10.1152/ajplung.1992.263.5.L546
82. Alcorn JL, Hammer RE, Graves KR, Smith ME, Maika SD, Michael LF, et al. Analysis of genomic regions involved in regulation of the rabbit *surfactant protein A* gene in transgenic mice. *Am J Physiol Lung Cell Mol Physiol*. (1999) 277:L349–61. doi: 10.1152/ajplung.1999.277.2.L349
83. Kim CJ, Kim JS, Kim YM, Cushenberry E, Richani K, Espinoza J, et al. Fetal macrophages are not present in the myometrium of women with labor at term. *Am J Obstet Gynecol*. (2006) 195:829–33. doi: 10.1016/j.ajog.2006.06.052
84. Lee DC, Romero R, Kim CJ, Chaiworapongsa T, Tarca AL, Lee J, et al. Surfactant protein-A as an anti-inflammatory component in the amnion: implications for human pregnancy. *J Immunol*. (2010) 184:6479–91. doi: 10.4049/jimmunol.0903867
85. Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, et al. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell*. (2003) 115:13–23. doi: 10.1016/S0092-8674(03)00758-X
86. Murakami S, Iwaki D, Mitsuzawa H, Sano H, Takahashi H, Voelker DR, et al. Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-α secretion in U937 cells and alveolar macrophages by direct interaction with toll-like receptor 2. *J Biol Chem*. (2002) 277:6830–7. doi: 10.1074/jbc.M106671200

87. Sato M, Sano H, Iwaki D, Kudo K, Konishi M, Takahashi H, et al. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappaB activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J Immunol*. (2003) 171:417–25. doi: 10.4049/jimmunol.171.1.417
88. Ohya M, Nishitani C, Sano H, Yamada C, Mitsuzawa H, Shimizu T, et al. Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 through the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide. *Biochemistry*. (2006) 45:8657–64. doi: 10.1021/bi060176z
89. Tsan MF, Gao B. Endogenous ligands of Toll-like receptors. *J Leukoc Biol*. (2004) 76:514–9. doi: 10.1189/jlb.0304127
90. Shynlova O, Tsui P, Jaffer S, Lye SJ. Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. *Eur J Obstet Gynecol Reprod Biol*. (2009) 144(Suppl 1):S2–10. doi: 10.1016/j.ejogrb.2009.02.044
91. Wu X, Morgan KG, Jones CJ, Tribe RM, Taggart MJ. Myometrial mechanoadaptation during pregnancy: implications for smooth muscle plasticity and remodelling. *J Cell Mol Med*. (2008) 12:1360–73. doi: 10.1111/j.1582-4934.2008.00306.x
92. Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell*. (2006) 125:411–4. doi: 10.1016/j.cell.2006.04.021
93. Liu D, Benlhabib H, Mendelson CR. cAMP enhances estrogen-related receptor alpha (ERRalpha) transcriptional activity at the *SP-A* promoter by increasing its interaction with protein kinase A and steroid receptor coactivator 2 (SRC-2). *Mol Endocrinol*. (2009) 23:772–83. doi: 10.1210/me.2008-0282
94. Yi M, Tong GX, Murry B, Mendelson CR. Role of CBP/p300 and SRC-1 in transcriptional regulation of the pulmonary surfactant protein-A (SP-A) gene by thyroid transcription factor-1 (TTF-1). *J Biol Chem*. (2001) 277:2997–3005. doi: 10.1074/jbc.M109793200
95. Islam KN, Mendelson CR. Permissive effects of oxygen on cyclic AMP and interleukin-1 stimulation of surfactant protein A gene expression are mediated by epigenetic mechanisms. *Mol Cell Biol*. (2006) 26:2901–12. doi: 10.1128/MCB.26.8.2901-2912.2006
96. Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. *Nat Rev Cancer*. (2009) 9:615–30. doi: 10.1038/nrc2695
97. Johnson AB, O'Malley BW. Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. *Mol Cell Endocrinol*. (2012) 348:430–9. doi: 10.1016/j.mce.2011.04.021
98. Mark M, Yoshida-Komiya H, Gehin M, Liao L, Tsai MJ, O'Malley BW, et al. Partially redundant functions of SRC-1 and TIF2 in postnatal survival and male reproduction. *Proc Natl Acad Sci USA*. (2004) 101:4453–8. doi: 10.1073/pnas.0400234101
99. Hoffman DR, Romero R, Johnston JM. Detection of platelet-activating factor in amniotic fluid of complicated pregnancies. *Am J Obstet Gynecol*. (1990) 162:525–8. doi: 10.1016/0002-9378(90)90423-5
100. Yasuda K, Furukawa M, Johnston JM. Effect of estrogens on plasma platelet-activating factor acetylhydrolase and the timing of parturition in the rat. *Biol Reprod*. (1996) 54:224–9. doi: 10.1095/biolreprod54.1.224
101. Zhu YP, Hoffman DR, Hwang SB, Miyaura S, Johnston JM. Prolongation of parturition in the pregnant rat following treatment with a platelet activating factor receptor antagonist. *Biol Reprod*. (1991) 44:39–42. doi: 10.1095/biolreprod44.1.39
102. Ishii S, Kuwaki T, Nagase T, Maki K, Tashiro F, Sunaga S, et al. Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. *J Exp Med*. (1998) 187:1779–88. doi: 10.1084/jem.187.11.1779
103. Jeanneton O, Delvaux M, Botella A, Frexinos J, Bueno L. Platelet-activating factor (PAF) induces a contraction of isolated smooth muscle cells from guinea pig ileum: intracellular pathway involved. *J Pharmacol Exp Ther*. (1993) 267:31–7.
104. Kim BK, Ozaki H, Lee SM, Karaki H. Increased sensitivity of rat myometrium to the contractile effect of platelet activating factor before delivery. *Br J Pharmacol*. (1995) 115:1211–4. doi: 10.1111/j.1476-5381.1995.tb15027.x
105. Montrucchio G, Alloatti G, Tetta C, Roffinello C, Emanuelli G, Camussi G. *In vitro* contractile effect of platelet-activating factor on guinea-pig myometrium. *Prostaglandins*. (1986) 32:539–54. doi: 10.1016/0090-6980(86)90036-5
106. Tetta C, Montrucchio G, Alloatti G, Roffinello C, Emanuelli G, Benedetto C, et al. Platelet-activating factor contracts human myometrium *in vitro*. *Proc Soc Exp Biol Med*. (1986) 83:376–81. doi: 10.3181/00379727-183-42435
107. Bridges JP, Ikegami M, Brilli LL, Chen X, Mason RJ, Shannon JM. LPCAT1 regulates surfactant phospholipid synthesis and is required for transitioning to air breathing in mice. *J Clin Invest*. (2010) 120:1736–48. doi: 10.1172/JCI38061
108. Chen X, Hyatt BA, Mucenski ML, Mason RJ, Shannon JM. Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells. *Proc Natl Acad Sci USA*. (2006) 103:11724–9. doi: 10.1073/pnas.0604946103
109. Nakanishi H, Shindou H, Hishikawa D, Harayama T, Ogasawara R, Suwabe A, et al. Cloning and characterization of mouse lung-type acyl-CoA:lysophosphatidylcholine acyltransferase 1 (LPCAT1). Expression in alveolar type II cells and possible involvement in surfactant production. *J Biol Chem*. (2006) 281:20140–7. doi: 10.1074/jbc.M600225200
110. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone receptor (PR) plays a major anti-inflammatory role in human myometrial cells by antagonism of NF-kappaB activation of cyclooxygenase 2 (COX-2) expression. *Mol Endocrinol*. (2006) 20:2724–33. doi: 10.1210/me.2006-0112
111. Dong X, Yu C, Shynlova O, Challis JR, Rennie PS, Lye SJ. p54nrb is a transcriptional corepressor of the progesterone receptor that modulates transcription of the labor-associated gene, connexin 43 (Gja1). *Mol Endocrinol*. (2009) 23:1147–60. doi: 10.1210/me.2008-0357
112. Dong X, Shylnova O, Challis JR, Lye SJ. Identification and characterization of the protein-associated splicing factor as a negative co-regulator of the progesterone receptor. *J Biol Chem*. (2005) 280:13329–40. doi: 10.1074/jbc.M409187200
113. Deroo BJ, Archer TK. Differential activation of the IκBα and mouse mammary tumor virus promoters by progesterone and glucocorticoid receptors. *J Steroid Biochem Mol Biol*. (2002) 81:309–17. doi: 10.1016/S0960-0760(02)00072-9
114. Chen CC, Hardy DB, Mendelson CR. Progesterone receptor inhibits proliferation of human breast cancer cells via induction of MAPK phosphatase 1 (MKP-1/DUSP1). *J Biol Chem*. (2011) 286:43091–102. doi: 10.1074/jbc.M111.295865
115. Vicent GP, Ballare C, Nacht AS, Clausell J, Subtil-Rodriguez A, Quiles I, et al. Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3. *Mol Cell*. (2006) 24:367–81. doi: 10.1016/j.molcel.2006.10.011
116. Spoelstra NS, Manning NG, Higashi Y, Darling D, Singh M, Shroyer KR, et al. The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res*. (2006) 66:3893–902. doi: 10.1158/0008-5472.CAN-05-2881
117. Hardy DB, Mendelson CR. Progesterone receptor (PR) antagonism of the inflammatory signals leading to labor. *Fetal Maternal Med Rev*. (2006) 17:281–9. doi: 10.1017/S0965539506001811
118. Siiteri PK, Stites DP. Immunologic and endocrine interrelationships in pregnancy. *Biol Reprod*. (1982) 26:1–14. doi: 10.1095/biolreprod26.1.1
119. Tan H, Yi L, Rote NS, Hurd WW, Mesiano S. Progesterone receptor-A and -B have opposite effects on proinflammatory gene expression in human myometrial cells: implications for progesterone actions in human pregnancy and parturition. *J Clin Endocrinol Metab*. (2012) 97:E719–30. doi: 10.1210/jc.2011-3251
120. Shynlova O, Lee YH, Srikhajon K, Lye SJ. Physiologic uterine inflammation and labor onset: integration of endocrine and mechanical signals. *Reprod Sci*. (2013) 20:154–67. doi: 10.1177/1933719112446084
121. Havelock JC, Keller P, Muleba N, Mayhew BA, Casey BM, Rainey WE, et al. Human myometrial gene expression before and during parturition. *Biol Reprod*. (2005) 72:707–19. doi: 10.1095/biolreprod.104.032979
122. Kalkhoven E, Wissink S, van der Saag PT, van der BB. Negative interaction between the RelA(p65) subunit of NF-κB and the progesterone receptor. *J Biol Chem*. (1996) 271:6217–24. doi: 10.1074/jbc.271.11.6217

123. Baldwin ASJ. The NF-kappaB and IkappaB proteins: new discoveries and insights. *Annu Rev Immunol*. (1996) 14:649–83. doi: 10.1146/annurev.immunol.14.1.649
124. Miller L, Hunt JS. Regulation of TNF-alpha production in activated mouse macrophages by progesterone. *J Immunol*. (1998) 160:5098–104.
125. Hardy DB, Janowski BA, Chen, CC, Mendelson CR. Progesterone receptor inhibits aromatase and inflammatory response pathways in breast cancer cells via ligand-dependent and ligand-independent mechanisms. *Mol Endocrinol*. (2008) 22:1812–24. doi: 10.1210/me.2007-0443
126. Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, et al. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem*. (1998) 273:3285–90. doi: 10.1074/jbc.273.6.3285
127. Gupta SC, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta*. (2010) 1799:775–87. doi: 10.1016/j.bbagrm.2010.05.004
128. Kyriakis JM. Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Expr*. (1999) 7:217–31.
129. Mitchell JA, Lye SJ. Differential activation of the connexin 43 promoter by dimers of activator protein-1 transcription factors in myometrial cells. *Endocrinology*. (2005) 146:2048–54. doi: 10.1210/en.2004-1066
130. Nadeem L, Shynlova O, Matysiak-Zablocki E, Mesiano S, Dong X, Lye S. Molecular evidence of functional progesterone withdrawal in human myometrium. *Nat Commun*. (2016) 7:11565. doi: 10.1038/ncomms11565
131. Williams KC, Renthal NE, Condon JC, Gerard RD, Mendelson CR. MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor. *Proc Natl Acad Sci USA*. (2012) 109:7529–34. doi: 10.1073/pnas.1200650109
132. Peters GA, Yi L, Skomorovska-Prokvolit Y, Patel B, Amini P, Tan H, et al. Inflammatory stimuli increase progesterone receptor-A stability and transrepressive activity in myometrial cells. *Endocrinology*. (2016) 58:en2016-1537. doi: 10.1210/en.2016-1537
133. Amini P, Michniuk D, Kuo K, Yi L, Skomorovska-Prokvolit Y, Peters GA, et al. Human parturition involves phosphorylation of progesterone receptor-A at serine-345 in myometrial cells. *Endocrinology*. (2016) 157:4434–45. doi: 10.1210/en.2016-1654
134. Cochrane DR, Spoelstra NS, Richer JK. The role of miRNAs in progesterone action. *Mol Cell Endocrinol*. (2012) 357:50–9. doi: 10.1016/j.mce.2011.09.022
135. Brabletz S, Bajdak K, Meidhof S, Burk U, Niedermann G, Firat E, et al. The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. *EMBO J*. (2011) 30:770–82. doi: 10.1038/emboj.2010.349
136. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res*. (2008) 68:7846–54. doi: 10.1158/0008-5472.CAN-08-1942
137. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep*. (2008) 9:582–9. doi: 10.1038/embor.2008.74
138. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et a. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol*. (2008) 10:593–601. doi: 10.1038/ncb1722
139. Loebel DA, Tsoi B, Wong N, Tam PP. A conserved noncoding intronic transcript at the mouse Dnm3 locus. *Genomics*. (2005) 85:782–9. doi: 10.1016/j.ygeno.2005.02.001
140. Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H, Dey SK. MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci USA*. (2007) 104:15144–9. doi: 10.1073/pnas.0705917104
141. Puri CP, Garfield RE. Changes in hormone levels and gap junctions in the rat uterus during pregnancy and parturition. *Biol Reprod*. (1982) 27:967–75. doi: 10.1095/biolreprod27.4.967
142. Power SG, Challis JR. The effects of gestational age and intrafetal ACTH administration on the concentration of progesterone in the fetal membranes, endometrium, and myometrium of pregnant sheep. *Can J Physiol Pharmacol*. (1987) 65:136–40. doi: 10.1139/y87-027
143. Csapo AI, Eskola J, Tarro S. Gestational changes in the progesterone and prostaglandin F levels of the guinea-pig. *Prostaglandins*. (1981) 21:53–64. doi: 10.1016/0090-6980(81)90196-9
144. Runnebaum B, Zander J. Progesterone and 20alpha-dihydroprogesterone in human myometrium during pregnancy. *Acta Endocrinol Suppl*. (1971) 150:3–45. doi: 10.1530/acta.0.066S005
145. Richer JK, Lange CA, Manning NG, Owen G, Powell R, Horwitz KB. Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. *J Biol Chem*. (1998) 273:31317–26. doi: 10.1074/jbc.273.47.31317
146. Shynlova O, Oldenhof A, Dorogin A, Xu Q, Mu J, Nashman N, et al. Myometrial apoptosis: activation of the caspase cascade in the pregnant rat myometrium at midgestation. *Biol Reprod*. (2006) 74:839–49. doi: 10.1095/biolreprod.105.048124
147. Jeyasuria P, Wetzel J, Bradley M, Subedi K, Condon JC. Progesterone-regulated caspase 3 action in the mouse may play a role in uterine quiescence during pregnancy through fragmentation of uterine myocyte contractile proteins. *Biol Reprod*. (2009) 80:928–34. doi: 10.1095/biolreprod.108.070425
148. Kyathanahalli C, Organ K, Moreci RS, Anamthathmakula P, Hassan SS, Caritis SN, et al. Uterine endoplasmic reticulum stress-unfolded protein response regulation of gestational length is caspase-3 and−7-dependent. *Proc Natl Acad Sci USA*. (2015) 112:14090–5. doi: 10.1073/pnas.1518309112
149. Suresh A, Subedi K, Kyathanahalli C, Jeyasuria P, Condon JC. Uterine endoplasmic reticulum stress and its unfolded protein response may regulate caspase 3 activation in the pregnant mouse uterus. *PLoS ONE*. (2013) 8:e75152. doi: 10.1371/journal.pone.0075152
150. Wei LL, Norris BM, Baker CJ. An N-terminally truncated third progesterone receptor protein, PR(C), forms heterodimers with PR(B) but interferes in PR(B)-DNA binding. *J Steroid Biochem Mol Biol*. (1997) 62:287–97. doi: 10.1016/S0960-0760(97)00044-7
151. Wei LL, Gonzalez-Aller C, Wood WM, Miller LA, Horwitz KB. 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. *Mol Endocrinol*. (1990) 4:1833–40. doi: 10.1210/mend-4-12-1833
152. Wei LL, Hawkins P, Baker C, Norris B, Sheridan PL, Quinn PG. An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. *Mol Endocrinol*. (1996) 10:1379–87. doi: 10.1210/me.10.11.1379
153. Samalecos A, Gellersen B. Systematic expression analysis and antibody screening do not support the existence of naturally occurring progesterone receptor (PR)-C, PR-M, or other truncated PR isoforms. *Endocrinology*. (2008) 149:5872–87. doi: 10.1210/en.2008-0602
154. Li X, Wong J, Tsai SY, Tsai MJ, O'Malley BW. Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. *Mol Cell Biol*. (2003) 23:3763–73. doi: 10.1128/MCB.23.11.3763-3773.2003
155. Mukherjee A, Soyal SM, Fernandez-Valdivia R, Gehin M, Chambon P, DeMayo FJ, et al. Steroid receptor coactivator 2 is critical for progesterone-dependent uterine function and mammary morphogenesis in the mouse. *Mol Cell Biol*. (2006) 26:6571–83. doi: 10.1128/MCB.00654-06
156. Condon JC, Jeyasuria P, Faust JM, Wilson JM, Mendelson CR. A decline in progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of labor. *Proc Natl Acad Sci USA*. (2003) 100:9518–23. doi: 10.1073/pnas.1633616100
157. Lindstrom TM, Mohan AR, Johnson MR, Bennett PR. Histone deacetylase inhibitors exert time-dependent effects on nuclear factor-κB but consistently suppress the expression of proinflammatory genes in human myometrial cells. *Mol Pharmacol*. (2008) 74:109–21. doi: 10.1124/mol.107.042838
158. Moynihan AT, Hehir MP, Sharkey AM, Robson SC, Europe-Finner GN, Morrison JJ. Histone deacetylase inhibitors and a functional potent inhibitory effect on human uterine contractility. *Am J Obstet Gynecol*. (2008) 199:167. doi: 10.1016/j.ajog.2008.01.002
159. Leite RS, Brown AG, Strauss JF, III. Tumor necrosis factor-α suppresses the expression of steroid receptor coactivator-1 and−2: a possible mechanism contributing to changes in steroid hormone responsiveness. *FASEB J*. (2004) 18:1418–20. doi: 10.1096/fj.04-1684fje

160. Mendelson CR. Minireview: fetal-maternal hormonal signaling in pregnancy and labor. *Mol Endocrinol.* (2009) 23:947–54. doi: 10.1210/me.2009-0016
161. Ishida M, Choi JH, Hirabayashi K, Matsuwaki T, Suzuki M, Yamanouchi K, et al. Reproductive phenotypes in mice with targeted disruption of the 20alpha-hydroxysteroid dehydrogenase gene. *J Reprod Dev*. (2007) 53:499–508. doi: 10.1262/jrd.18125
162. Haraguchi H, Saito-Fujita T, Hirota Y, Egashira M, Matsumoto L, Matsuo M, et al. MicroRNA-200a locally attenuates progesterone signaling in the cervix, preventing embryo implantation. *Mol Endocrinol.* (2014) 28:1108–17. doi: 10.1210/me.2014-1097

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 12 December 2019
doi: 10.3389/fendo.2019.00869

CRF_1 Receptor Signaling via the ERK1/2-MAP and Akt Kinase Cascades: Roles of Src, EGF Receptor, and PI3-Kinase Mechanisms

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 19 April 2019
Accepted: 27 November 2019
Published: 12 December 2019

Citation:
Parra-Mercado GK, Fuentes-Gonzalez AM, Hernandez-Aranda J, Diaz-Coranguez M, Dautzenberg FM, Catt KJ, Hauger RL and Olivares-Reyes JA (2019) CRF_1 Receptor Signaling via the ERK1/2-MAP and Akt Kinase Cascades: Roles of Src, EGF Receptor, and PI3-Kinase Mechanisms. Front. Endocrinol. 10:869. doi: 10.3389/fendo.2019.00869

In the present study, we determined the cellular regulators of ERK1/2 and Akt signaling pathways in response to human CRF_1 receptor (CRF_1R) activation in transfected COS-7 cells. We found that Pertussis Toxin (PTX) treatment or sequestering Gβγ reduced CRF_1R-mediated activation of ERK1/2, suggesting the involvement of a G_i-linked cascade. Neither G_s/PKA nor G_q/PKC were associated with ERK1/2 activation. Besides, CRF induced EGF receptor (EGFR) phosphorylation at Tyr^{1068}, and selective inhibition of EGFR kinase activity by AG1478 strongly inhibited the CRF_1R-mediated phosphorylation of ERK1/2, indicating the participation of EGFR transactivation. Furthermore, CRF-induced ERK1/2 phosphorylation was not altered by pretreatment with batimastat, GM6001, or an HB-EGF antibody indicating that metalloproteinase processing of HB-EGF ligands is not required for the CRF-mediated EGFR transactivation. We also observed that CRF induced Src and PYK2 phosphorylation in a Gβγ-dependent manner. Additionally, using the specific Src kinase inhibitor PP2 and the dominant-negative-SrcYF-KM, it was revealed that CRF-stimulated ERK1/2 phosphorylation depends on Src activation. PP2 also blocked the effect of CRF on Src and EGFR (Tyr^{845}) phosphorylation, further demonstrating the centrality of Src. We identified the formation of a protein complex consisting of CRF_1R, Src, and EGFR facilitates EGFR transactivation and CRF_1R-mediated signaling. CRF stimulated Akt phosphorylation, which was dependent on G_i/βγ subunits, and Src activation, however, was only slightly dependent on EGFR transactivation. Moreover, PI3K inhibitors were able to inhibit not only the CRF-induced phosphorylation of Akt, as expected, but also ERK1/2 activation by CRF suggesting a PI3K dependency in the CRF_1R ERK signaling. Finally, CRF-stimulated ERK1/2 activation was similar in the wild-type CRF_1R and the phosphorylation-deficient CRF_1R-Δ386 mutant, which has impaired agonist-dependent β-arrestin-2 recruitment; however, this situation

may have resulted from the low β-arrestin expression in the COS-7 cells. When β-arrestin-2 was overexpressed in COS-7 cells, CRF-stimulated ERK1/2 phosphorylation was markedly upregulated. These findings indicate that on the base of a constitutive CRF_1R/EGFR interaction, the $G_i/\beta\gamma$ subunits upstream activation of Src, PYK2, PI3K, and transactivation of the EGFR are required for CRF_1R signaling via the ERK1/2-MAP kinase pathway. In contrast, Akt activation via CRF_1R is mediated by the Src/PI3K pathway with little contribution of EGFR transactivation.

Keywords: corticotropin-releasing factor, CRF_1 receptor, EGF receptor transactivation, ERK1/2, Src, PI3K/Akt

INTRODUCTION

Behavioral, cognitive, neuroendocrine, and autonomic responses to stress are regulated by CRF_1 and CRF_2 receptors (CRF_1R and CRF_2R) (1–3). The preferred mode of signal transduction by both CRF receptors was initially believed to be activation of the G_s/adenylyl cyclase/PKA signaling pathway (1–3). Subsequently, CRF_1R and CRF_2R were also found to signal *via* the PLC/PKC cascade stimulating intracellular calcium mobilization and IP3 formation (1–4). Besides, both CRF receptors can activate mitogen-activated protein (MAP) kinase cascades in neuronal, cardiac, and myometrial cells endogenously expressing CRF_1R or CRF_2R and in recombinant cell lines expressing either receptor (2, 3, 5, 6). Several reports suggested that cellular background directed CRF_1R to signal selectively via a specific MAP kinase pathway. For example, agonist-activated CRF_1Rs stimulated phosphorylation of ERK1/2 and p38 MAP kinases in PC12 and fetal microglial cells (7, 8) while CRF_1Rs activated ERK1/2 but not JNK and p38 in CHO cells (9). In human mast cells and HaCaT keratinocytes, on the other hand, CRF_1Rs induce phosphorylation of p38 but not ERK or JNK MAP kinases (10, 11). Most studies suggest, however, that the ERK1/2 cascade is the MAP kinase pathway preferentially used by CRF receptors (5, 9, 12, 13).

Signaling via the cyclic AMP (cAMP)-PKA pathway by G_s-coupled GPCRs has been proposed to mediate upstream activation of the ERK cascade in cells with high B-Raf expression (14). Consistent with this concept, PKA regulates CRF_1R-mediated ERK activation and ERK-dependent Elk1 transcription in AtT-20 pituitary cells that express high B-Raf levels (15). Kageyama et al. (16) found, however, that ERK activation by CRF_1R was mediated by a PKA-independent mechanism in AtT-20 cells. Moreover, other studies have reported that PKA does not play a role in CRF_1R ERK signaling in rat CATH.a and rat fetal microglial cells, locus coeruleus neurons, and transfected CHO cells (8, 9, 12, 17). CRF_1R can also activate the ERK1/2 cascade via a PKC-dependent mechanism, based on data showing that pretreatment with a PLC or PKC inhibitor blocked urocortin 1 (Ucn1)-stimulated phosphorylation of ERK1/2 in CRF_1R-expressing human myometrial, CHO, and HEK293 cells (12, 13), and in rat hippocampal neurons (18). PKC inhibitor pretreatment, however, failed to block CRF- and Ucn1-stimulated ERK1/2 phosphorylation in CRF_1R-expressing pituitary AtT20 cells and brain-derived CATH.a cells expressing both CRF receptors (12, 16). These findings suggest that cellular background may also govern the ability of PKA or PKC pathways to regulate CRF_1R ERK1/2 signaling similar to its possible role in mediating CRF_1R selective activation of a specific MAP kinase cascade.

MEK1/2-mediated phosphorylation of ERK1/2 at Thr^{202} and Tyr^{204} during CRF_1R and CRF_2R signaling in various cell lines has been confirmed by inhibiting ERK1/2 activation with PD98059 (2, 9, 12, 13, 19). Inhibiting C-Raf function by pretreatment with R1-K1 inhibitor or blocking Ras activation by transfection with the dominant-negative mutant RasS17N inhibited Ucn1-stimulated ERK1/2 phosphorylation in CRF_1R-expressing CHO and HEK293 cells (5, 12). CRF_2R activation by urocortin 2 (Ucn2) and urocortin 3 (Ucn3) has also been shown to signal via the Ras→C-Raf→MEK1/2 cascade in rat cardiomyocytes, based on the ability of manumycin A (a Ras inhibitor) and R1-K1 to abolish ERK1/2 phosphorylation (19). Other research has provided evidence for a phosphoinositide 3-kinase (PI3K)-dependent mechanism contributing to CRF_1R- and CRF_2R-mediated ERK1/2 activation in HEK293, CHO, A7r5, and CATH.a cells (5, 9, 12). EGF receptor (EGFR) transactivation, possibly by matrix metalloproteinase (MMP)-mediated ligand release, has been shown to contribute to Ucn1-stimulated ERK1/2 phosphorylation in HEK293 cells, although the mechanisms for CRF_1R-mediated transactivation of the EGFR were not determined (5). Furthermore, another study reported that CRF receptor ERK signaling in the mouse atrial HL-1 cardiomyocyte line involved activation of Src (20).

In addition, activation of CRF_1R or $CRF_{2(b)}R$ can stimulate phosphorylation of Akt (5, 21). $CRF_{2(b)}R$ Akt signaling in HEK293 cells is mediated by pertussis-sensitive G proteins and PI3K but not by cAMP-stimulated activation of PKA or EPAC, or by PKC (21). The mechanisms regulating Akt signal transduction by CRF_1R, however, have not been investigated. Because upstream kinase pathway mediation of CRF_1R signal transduction via the ERK and Akt cascades are not well-understood, the primary goal of this study was to test the hypothesis that Src tyrosine kinase and EGFR transactivation

Abbreviations: CRF, Corticotropin-releasing factor; CRF_1R, corticotropin-releasing factor receptor type 1; ct-βARK, β-adrenergic receptor kinase carboxyl terminus peptide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellularly regulated kinases 1 and 2; GPCR, G protein-coupled receptor; MMP, matrix metalloproteinase; MAP kinase, mitogen-activated protein kinase; PYK2, proline-rich tyrosine kinase 2; PI3K, phosphatidylinositol 3-kinase; c-Src, human homolog of the v-Src Rous sarcoma proto-oncogene; Ucn, urocortin.

are essential regulators of these CRF_1R signaling pathways. We also sought to determine the relative importance of G protein βγ subunits, second messenger kinases, and PI3K in the activation of the ERK1/2 and Akt cascades by the CRF_1R. The results of our study indicate that upstream utilization of Src and PI3K are involved in ERK and Akt signal transduction by the agonist-activated CRF_1R in COS-7 cells, without mediation by PKA and PKC, while transactivation of the EGFR is mainly required for CRF_1R to stimulate phosphorylation of ERK but not for Akt activation.

MATERIALS AND METHODS

Materials

General reagents utilized were as follows: (i) DMEM, fetal bovine serum (FBS), antibiotic solutions and other cell culture reagents from Invitrogen/GIBCO (Carlsbad, CA); (ii) Pertussis Toxin, reagents for electrophoresis and other highly pure chemicals from Sigma-Aldrich (St. Louis, MO). Human/rat CRF was purchased from Bachem (Torrance, CA). Phorbol 12-myristate 13-acetate (PMA), and the following specific inhibitors were purchased from Calbiochem (La Jolla, CA): Src inhibitor PP2; EGFR tyrosine kinase inhibitor AG1478; PKA inhibitor H89; PKC inhibitor bisindolylmaleimide I, BIM; PI3K inhibitors wortmannin and LY294002; MMP inhibitor GM6001, and Protease Inhibitor Cocktail Set III. BB-94 (batimastat) was obtained from British Biotechnology Ltd (Oxford, UK). Antibodies for Western blots were obtained from the following sources: (i) phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}), total ERK1/2, total EGFR and phospho-c-Src (Tyr^{416}) from Cell Signaling Technology (Beverly, MA); (ii) total ERK2, total c-Src and total Akt from Santa Cruz Biotechnology (Santa Cruz, CA); (iii) phospho-EGFR (Tyr^{1068}), phospho-EGFR (Tyr^{1173}) and phospho-EGFR (Tyr^{845}) from Biosource-Invitrogen (Carlsbad, CA); (iv) phospho-Akt (Ser^{473}) from Biosource International (Camarillo, CA); (v) phospho-PYK2 (Tyr^{402}) from Calbiochem (La Jolla, CA); (vi) polyclonal anti-human HB-EGF antibody from R&D Systems (Minneapolis, MN); (vii) secondary antibodies conjugated to horseradish peroxidase from Zymed Laboratories (San Francisco, CA).

DNA Constructs

The HA-epitope tagging human CRF_1R (HA-CRF_1R), the HA-CRF_1R-Δ386 mutant, and the β-arrestin-2 constructs were previously described (22–24). Plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) was kindly provided by Dr. W. Koch (Center for Translational Medicine, Temple University, Philadelphia, PA), which is a scavenger for G protein βγ subunits (25). The expression vector pCEFL-SrcYF-KM, which contains the inactive form of SrcYF, SrcYF-KM (dominant-negative, dn-Src), was kindly provided by Dr. Silvio Gutkind (Department of Pharmacology, UCSD, La Jolla, CA) (26). Plasmid pUSEamp encoding dominant-negative Akt-K179M (dn-Akt) was from Upstate Biotechnology (Lake Placid, NY).

Cell Culture and Transfection

COS-7 cells (from the American Type Culture Collection) were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2, in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin (COS-7 growth medium). Transient transfections were performed using LipofectAMINE (Life Technologies: Gaithersburg, MD) as described previously (27). Cells were seeded at 8×10^5 cells/10-cm dish in COS-7 medium and cultured for 3 days before transfection. COS-7 cells were transfected in 5 ml/dish OptiMEM containing 10 μg/ml LipofectAMINE with empty vector, pcDNA3 encoding the HA-CRF_1R or the HA-CRF_1R-Δ386 mutant. In certain experiments, cells were co-transfected with plasmids containing: HA-CRF_1R and mock (empty vector); HA-CRF_1R and ct-βARK; HA-CRF_1R and dn-Src; HA-CRF_1R and dn-Akt, or HA-CRF_1R and full-length β-arrestin-2. After replacing the transfection medium with fresh growth medium, transfected COS-7 cells were cultured for 1 day. Subsequently, cells were re-seeded in 6-well plates and cultured for an additional day prior to the experiment.

Western Blot Methods

The protocols for measuring total and phosphorylated ERK1/2, c-Src, PYK2, Akt, and EGFR have been previously published (28, 29). After cells were cultured to 60–70% confluence, they were serum-deprived for 24 h. On the day of the experiment, cells were treated with the indicated ligands and inhibitors. No significant changes in the basal level of ERK1/2 or Akt phosphorylation were observed in cells pretreated with inhibitors, except for BIM, which showed a small increase in ERK1/2 activation (**Supplementary Figure S1**). After treatment, cells were placed on ice, the media was aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer 1X. The lysates were briefly sonicated, heated at 95°C for 5 min, and centrifuged for 5 min at 14,000 rpm. Resulting supernatants were loaded in separate lanes of a 10% SDS-PAGE gels and electrophoresed. Next, Western transfer on to PDVF membranes was completed. The Western blots were then probed with specific antibodies targeting phosphorylated and non-phosphorylated forms of ERK1/2, c-Src, PYK2, Akt, and EGFR for primary immunodetection. After blots were probed with horseradish peroxidase-conjugated secondary antibody, protein bands were visualized with enhanced chemiluminescence ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ or Pierce Biotechnology, Rockford, IL) and scanned using the GS-800 Calibrated Imaging Densitometer (Bio-Rad). The labeled bands were quantified using the Quantity One 4.6.3 software program (Bio-Rad).

Co-immunoprecipitation Assay

COS-7 cells transfected with HA-CRF_1R were grown in 10-cm dishes and serum-deprived for 24 h before treatment with 100 nM CRF for 10 min at 37°C. Cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM Orthovanadate, 1 mM NaF, 1% Nonidet P-40, 10% Glycerol, 2 mM EDTA, pH 7.4, containing protease inhibitors). After immunoprecipitation of HA-CRF_1Rs with anti-HA monoclonal antibody (HA.11;

Covance, San Diego, CA) and protein A/G PLUS-Agarose (Santa Cruz Biotechnology, CA), the proteins were resolved by SDS-PAGE, Western blotted, and probed with anti-EGFR polyclonal or anti-HA monoclonal antibodies, followed by a horseradish peroxidase conjugate to identify co-immunoprecipitated proteins. Blots were also stripped with stripping buffer (100 nM Glycine-HCl, pH 2.7) and reprobed with anti-c-Src polyclonal antibody. Western blot detection of co-immunoprecipitated Src was carried out as described above. Blots were visualized and quantified, as indicated above.

Statistical Analysis

Data are presented as mean ± S.E.M. Analyses of variances (ANOVAs) across experimental groups were performed using PRISM™, Version 8.0 for macOS (GraphPad Software, Inc., San Diego, CA). If the one-way ANOVA was statistically significant, planned *post-hoc* analyses were performed using Dunnet or Bonferroni's multiple comparison tests to determine individual group differences.

RESULTS

CRF-Induced ERK1/2 Phosphorylation Is Dependent on G_i Protein and the Gβγ Subunits

CRF treatment (100 nM) of COS-7 cells transiently transfected with HA-CRF_1R caused transient phosphorylation of ERK1/2 that reached a peak at 5–10 min and declined thereafter toward the basal level over the next 30 min (**Figure 1A**). CRF (100 nM) also caused time-dependent phosphorylation of ERK1/2 in CRF_1R-expressing HEK293 and CHO-K1 cells (data not shown), but the rate and magnitude of CRF-induced ERK1/2 activation was considerably less in these cell lines compared to COS-7 cells. In contrast, CRF_1Rs expressed in SK-N-MC neuroblastoma cells (4) failed to signal *via* the ERK1/2 cascade while fibroblast growth factor induced strong ERK1/2 phosphorylation in this cell line (**Supplementary Figure S2**). Therefore, all subsequent experiments studying ERK1/2 signaling were performed in COS-7 cells transfected with HA-CRF_1R cDNA. CRF-induced ERK1/2 activation was concentration-dependent, with a significant increase at 10 nM CRF (∼2.4-fold increase over control) and maximal effect over the 0.1–1 μM range (∼5.2-fold increase over control, **Figure 1B**). The EC_{50} was 25 nM and the maximum occurred at 100 nM for the CRF-induced ERK1/2 phosphorylation.

Most of the known actions of CRF_1Rs are mediated through the G_s/PKA signaling cascade, but some of the physiological actions of CRF are also known to occur through activation of G_q or G_i proteins (30). To determine the contributions of G_s/PKA-dependent mechanisms to MAP kinase activation, COS-7 cells were pretreated with the PKA inhibitor H89 (500 nM) for 30 min prior to stimulation with CRF (100 nM). As shown in **Figure 2A**, the PKA inhibitor failed to inhibit CRF-stimulated ERK1/2 phosphorylation. Furthermore, the magnitude of CRF_1R-mediated activation of ERK1/2 was similar in COS-7 cells pretreated for 30 min with the highly selective PKA inhibitor Rp-cAMP (0–100 μM) or vehicle (**Supplementary Figure S3**). We next explored the involvement of G_q/PKC in CRF_1R ERK signaling. A 30-min pretreatment of COS-7 cells with the PKC inhibitor BIM (1 μM), increased (∼1.6-fold increase over CRF stimulation) rather than decreased CRF-stimulated ERK1/2 activation (**Figure 2B**). In contrast, BIM pretreatment inhibited ERK1/2 phosphorylation resulting from PMA-induced PKC activation (**Figure 2B**). Thus, our data suggest that neither G_s/PKA nor G_q/PKC are required for the CRF_1R-mediated ERK1/2 signaling in COS-7 cells.

On the other hand, the release of Gβγ subunits during GPCR coupling to G protein, particularly through G_i, has an important role in downstream signaling in the ERK1/2 cascade (31, 32). Thus, we examined the role of G_i and Gβγ in CRF-stimulated ERK1/2 activation by two different experimental approaches: treatment with the G_i protein inhibitor pertussis toxin (PTX) and by co-transfecting COS-7 cells with plasmids encoding the carboxyl terminus of βARK containing its βγ-binding domain (ct-βARK) (**Supplementary Figure S4**), which sequesters βγ, and the CRF_1R. COS-7 cells expressing CRF_1Rs pretreated with 100 ng/ml PTX showed a marked reduction in CRF-induced ERK1/2 phosphorylation (**Figure 2C**), suggesting the coupling of CRF_1R to G_i to mediate ERK activation. Moreover, overexpressing ct-βARK in COS-7 cells co-expressing CRF_1Rs significantly reduced CRF-induced ERK1/2 phosphorylation by ∼40% (**Figure 2D**). Thus, our data implicate Gβγ subunits from PTX-sensitive heterotrimeric G proteins in CRF_1R-mediated activation of ERK1/2.

Transactivation of the EGFR During CRF_1R ERK1/2 Signaling

Because transactivation of receptor tyrosine kinases (RTKs), especially the EGFR, is often an important mechanism used by GPCRs to activate ERK1/2 (33, 34), we investigated the role of EGFR transactivation in CRF_1R-mediated ERK signaling. In COS-7 cells transiently transfected with HA-CRF_1R, EGF stimulation of the endogenous EGFRs caused ERK1/2 phosphorylation in a time-dependent manner, reaching a maximum effect after 5 min of stimulation, which persisted for at least 30 min (∼6.0-fold increase over time 0, **Figure 3A**). ERK1/2 phosphorylation was also increased by EGF (0–100 ng/ml) in a concentration-dependent manner (EC_{50} = 0.23 ng/ml, **Figure 3B**). Thus, these results are consistent with the well-established role of EGFR in ERK1/2 signaling (35).

When COS-7 cells expressing HA-CRF_1R were pretreated with the EGFR tyrosine kinase inhibitor AG1478 (100 nM, 30 min), a significant inhibition (∼80%) of CRF-induced maximal ERK1/2 phosphorylation was observed (**Figure 3C**). A concentration-dependent inhibition was observed with AG1478 concentrations of 0–1,000 nM with an IC_{50} of 10 nM (**Figure 3D**). Importantly, phosphorylation of the EGFR at Tyr^{1068} was detected with Western blots in COS-7 cells beginning at 2 min and becoming maximal at 5–10 min of CRF exposure (100 nM) (**Figure 3E**). Tyr^{1173} of the EGFR was phosphorylated

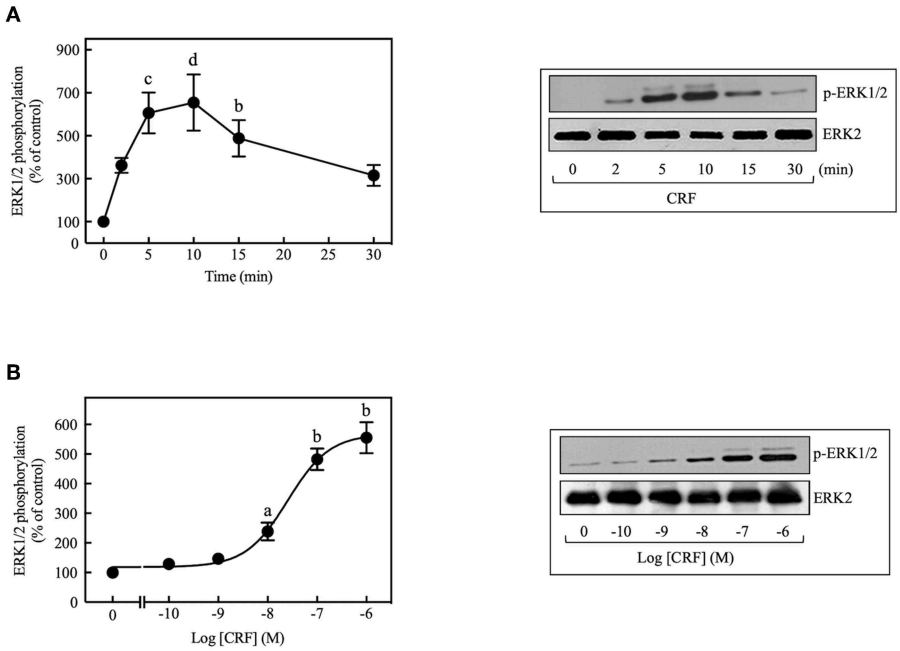

FIGURE 1 | CRF induces ERK1/2 phosphorylation in COS-7 expressing CRF_1Rs. **(A)** COS-7 cells expressing HA-CRF_1Rs were treated with 100 nM CRF for the indicated times. **(B)** COS-7 cells expressing HA-CRF_1Rs were exposed to the indicated concentration of CRF for 5 min. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-ERK1/2 Thr^{202}/Tyr^{204}, as described in Materials and Methods. ERK1/2 phosphorylation was quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Representative immunoblots are presented. Western blots were also probed for total ERK, showing equal loading. **(A)** $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$ vs. 0 min. **(B)** $^{a}p < 0.05$, $^{b}p < 0.01$ vs. 0 M.

in parallel with Tyr^{1068} in COS-7 cells stimulated with CRF (**Supplementary Figure S5**). Together, these results indicate that CRF-activated CRF_1R triggers phosphorylation of two critical amino acids located within the autophosphorylation loop that are required for EGFR activation (36, 37). Thus, CRF_1R signaling rapidly transactivates the EGFR, in agreement with a study reporting that Ucn1 stimulated EGFR transactivation in CRF_1R-expressing HEK293 cells (5).

MMPs catalyze the release of extracellular heparin-binding EGF (HB-EGF) ligand, which, in turn, binds to and activates the EGFR, thereby stimulating ERK1/2 phosphorylation (38–40). Although this process represents a significant mechanism for GPCR-mediated EGFR transactivation, we found that basal and CRF-stimulated ERK1/2 phosphorylation in transfected COS-7 cells was not altered by inhibiting the formation of the ligand HB-EGF with broad-spectrum MMP inhibitors batimastat BB-94 (5 μM) (**Figure 4A**) or GM6001 (0–20 μM) (**Figure 4B**). Similarly, blocking HB-EGF binding to the EGFR with an HB-EGF antibody (5 μg/ml) also failed to inhibit CRF-stimulated ERK1/2 phosphorylation (**Figure 4C**). In agreement with previous reports (39, 41), GM6001 pretreatment significantly attenuated ERK1/2 phosphorylation induced by PMA but not EGF (**Supplementary Figure S6**). Altogether, these results exclude a role of MMP in CRF-induced transactivation of the EGFR and subsequent phosphorylation of ERK1/2 in COS-7 cells.

Src Mediation of CRF_1R ERK1/2 Signaling

We then investigated the role of Src kinase, which can serve as an important upstream regulator of GPCR signaling via the ERK1/2 cascade (42, 43). Importantly, 100 nM CRF caused marked phosphorylation of Src at Tyr^{416}, which is a requirement for Src activation (44), reaching a maximum at 10 min (∼3.5-fold increase over time 0), and persisting for more than 30 min (**Figure 5A**). This activation was dependent on Gβγ release since ct-βARK expression reduced the CRF-induced Src phosphorylation (**Figure 5B**), and as expected, CRF-induced Src phosphorylation was prevented by pretreatment with the selective Src family kinase inhibitor PP2 (**Figure 9B**). To further evaluate the role of Src in CRF_1R ERK1/2 signaling, COS-7 cells were co-transfected with the CRF_1R and a dn-Src. Overexpression of inactive Src prevented ERK1/2 activation by CRF (**Figure 5C**). Other experiments demonstrated that PP2 pretreatment abolished CRF-stimulated ERK1/2 phosphorylation (**Figure 5D**), in a concentration-dependent manner (0–20 μM, $IC_{50} = 2\,\mu M$) (**Figure 5E**). These findings support our hypothesis that Src plays a central role in CRF_1R ERK1/2 signaling.

We next determined if CRF-stimulated Src activation is required for CRF_1R-induced transactivation of EGFRs. In this context, previous research has established that Src can activate EGFR signaling by phosphorylating Tyr^{845} of the EGFR protein (45, 46). As shown in **Figure 6A**, we found that 100 nM CRF stimulated in a time-dependent manner marked phosphorylation

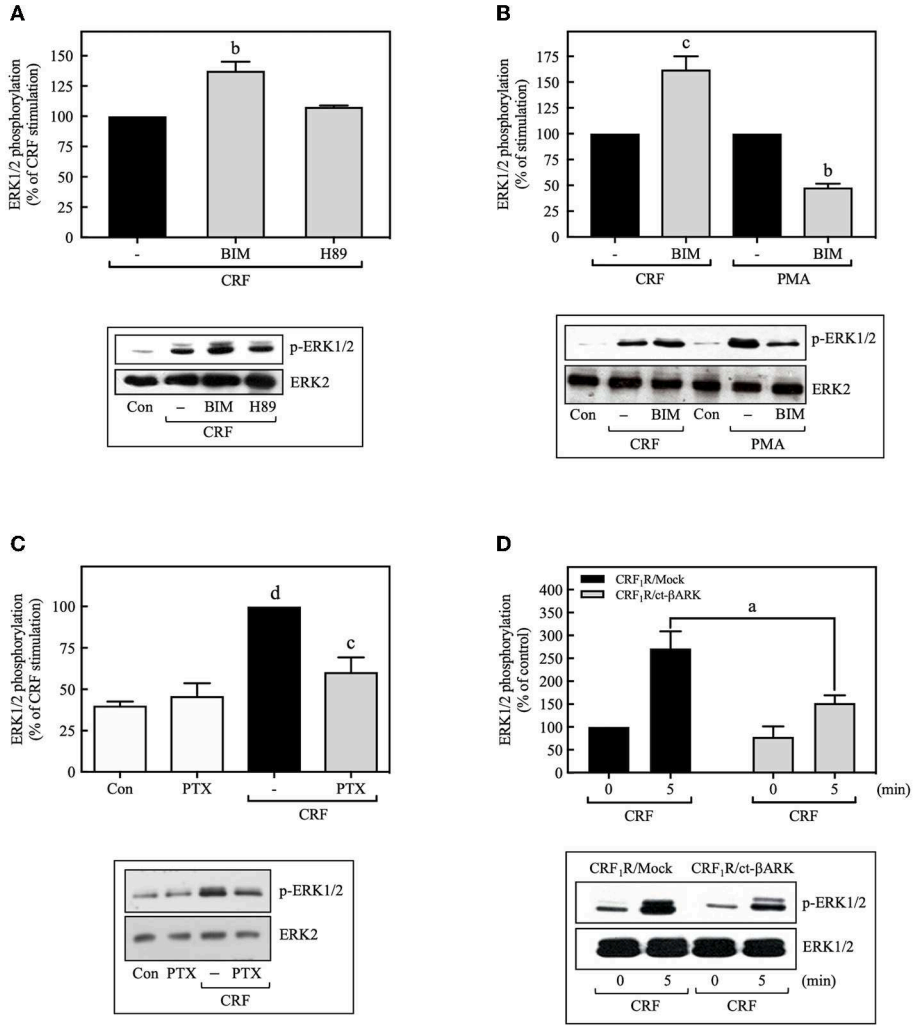

FIGURE 2 | CRF-induced ERK1/2 phosphorylation is dependent on G_i protein. COS-7 cells expressing HA-CRF_1Rs were pretreated with 0.5 μM H89 **(A)** or 1 μM BIM (bisindolylmaleimide I) **(A,B)** for 30 min, before stimulation with 100 nM CRF **(A,B)** for 5 min or 100 nM PMA **(B)** for 15 min. **(C)** COS-7 cells expressing HA-CRF_1Rs were pretreated with 100 ng/ml PTX for 15 h, before stimulation with 100 nM CRF for 5 min. **(D)** COS-7 cells co-transfected with a plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with 100 nM CRF for 5 min. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-ERK1/2 Thr^{202}/Tyr^{204}, as described in Materials and Methods. ERK1/2 phosphorylation was quantitated by densitometry, and mean values were plotted from five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total ERK showing equal loading. **(A)** $^b p < 0.01$ vs. CRF (–). **(B)** $^c p < 0.001$ vs. CRF (–); $^b p < 0.01$ vs. PMA (–). **(C)** $^d p < 0.0001$ vs. Con or PTX; $^c p < 0.001$ vs. CRF (–). **(D)** $^a p < 0.05$ vs. CRF_1R/Mock (5 min).

of EGFR at Tyr^{845} beginning at 2 min and becoming maximal at 10 min. This effect was blocked by pretreatment of the cells with PP2 (**Figure 6B**). In a recent study by Perkovska et al. (47), it was shown that V_{1b} vasopressin receptor interacts with Src at basal state, suggesting the formation of a GPCR/Src complex that facilitates MAP kinase activation. To evaluate if a CRF_1R/Src complex exists under basal conditions, we analyzed CRF_1R immunoprecipitates for the presence of co-precipitated Src under basal and CRF-stimulated conditions. As shown in **Figure 6C**, 100 nM CRF induced a robust interaction between the CRF_1R and Src after 10 min stimulation (∼8.0-fold increase over control). Interestingly, it was also observed that under the same immunoprecipitation conditions, the EGFR is also present in the CRF_1R/co-precipitated complex, even in the absence of CRF stimulation (**Figure 6D**). After stimulation with 100 nM CRF for 10 min, we observed a significant increase in the CRF_1R/EGFR interaction (∼2.5-fold increase over control). These observations suggest that CRF promotes the formation of a multiprotein complex that would allow rapid EGFR phosphorylation at Tyr^{845} by Src, present in this complex.

It has been shown that, in parallel to Src activation by many GPCRs, the proline-rich tyrosine kinase 2, PYK2, is also phosphorylated and activated, and in association with

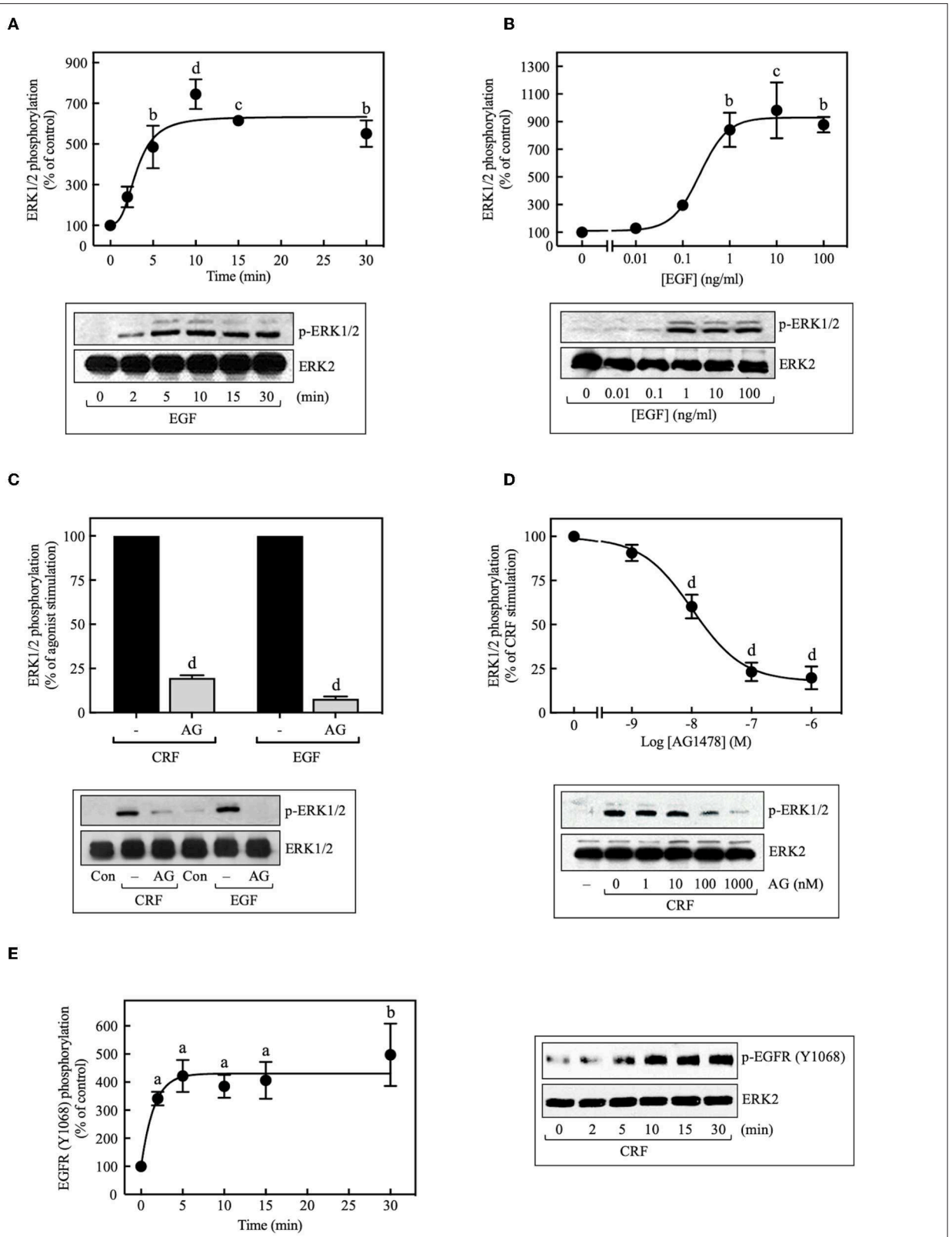

FIGURE 3 | Involvement of EGFR transactivation in CRF-stimulated ERK1/2 phosphorylation. COS-7 cells expressing HA-CRF_1Rs were treated with 10 ng/ml EGF for the indicated times **(A)** or exposed to the indicated concentration **(B)** of EGF for 10 min. COS-7 cells expressing HA-CRF_1Rs were pretreated with 100 nM **(C)** or

(Continued)

FIGURE 3 | the indicated concentrations of AG1478 (AG) **(D)** for 30 min, before stimulation with 100 nM CRF for 5 min **(C,D)** or 10 ng/ml EGF for 10 min **(C)**. **(E)** Effect of 100 nM CRF on EGFR phosphorylation at Tyr^{1068}. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-ERK1/2 Thr^{202}/Tyr^{204} **(A–D)** or anti-p-EGFR Tyr^{1068} **(E)**, as described in Materials and Methods. ERK1/2 and EGFR phosphorylation were quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total ERK, showing equal loading. **(A)** $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$ vs. 0 min. **(B)** $^{b}p < 0.01$, $^{c}p < 0.001$ vs. 0 ng/ml. **(C)** $^{d}p < 0.0001$ vs. CRF (–); $^{d}p < 0.0001$ vs. EGF (–). **(D)** $^{d}p < 0.0001$ vs. 0 M. **(E)** $^{a}p < 0.05$, $^{b}p < 0.01$ vs. 0 min.

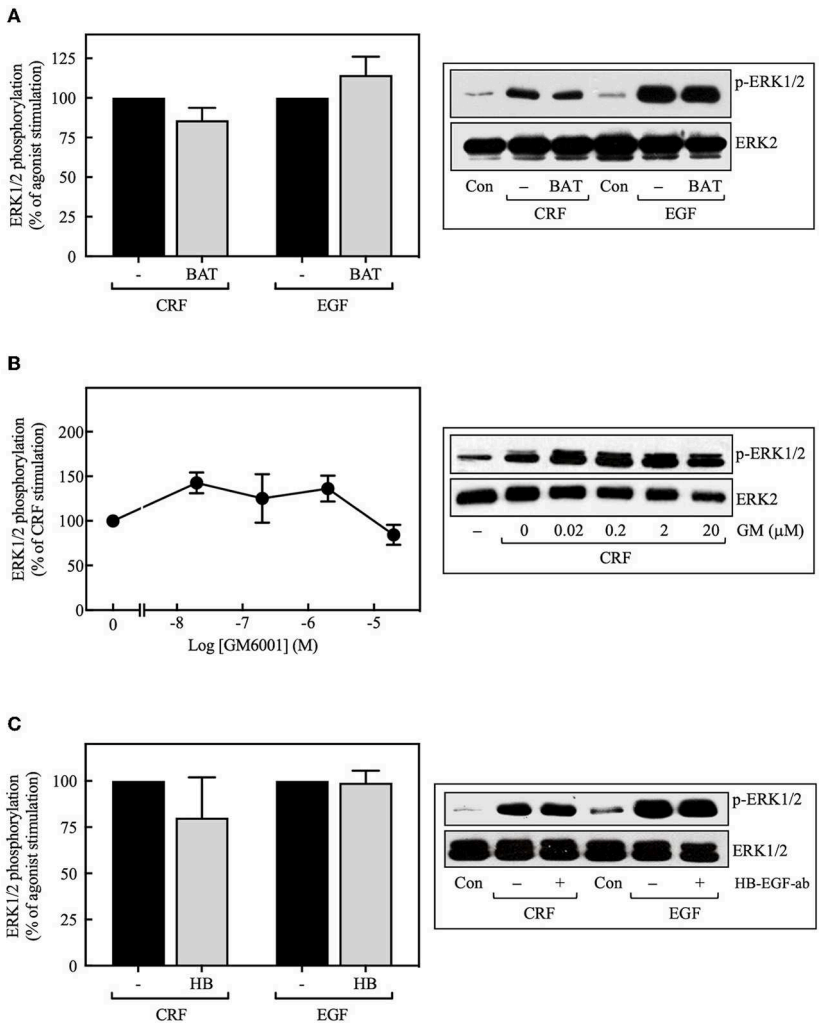

FIGURE 4 | Effect of the MMP inhibitors and the HB-EGF antibody on CRF-stimulated ERK1/2 phosphorylation. COS-7 cells expressing HA-CRF_1Rs were pretreated with 5 μM batimastat (BAT) **(A)**, the indicated concentrations of GM6001 (GM) **(B)** or 5 μg/ml HB-EGF antibody (HB) **(C)** for 30 min, before stimulation with 100 nM CRF for 5 min **(A–C)** or 10 ng/ml EGF for 10 min **(A,C)**. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-ERK1/2 Thr^{202}/Tyr^{204}, as described in Materials and Methods. ERK1/2 phosphorylation was quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total ERK showing equal loading.

Src is required for the subsequent transactivation of EGFR (44, 48). Therefore, we decided to assess whether activation of CRF_1R leads to PYK2 phosphorylation in COS-7 cells. As shown in **Figure 6E**, 100 nM CRF caused rapid phosphorylation of PYK2 in a time-dependent manner (0–30 min), reaching a maximum effect at 5 min and persisting for at least 30 min of stimulation. Interestingly, and as expected, CRF-mediated PYK2 phosphorylation was also dependent on Gβγ release (**Figure 5B**).

PI3K Mediation of CRF_1R ERK1/2 and Akt Signaling

PI3Ks can mediate important biological actions of GPCRs, including cell proliferation or survival, by serving as an upstream regulator of Akt and ERK cascades (49, 50). As shown in **Figure 7A**, 100 nM CRF caused rapid phosphorylation of Akt, an effect that was decreased by PTX pretreatment (**Figure 7B**) or ct-βARK overexpression (**Figure 7C**), similar to the previously observed effect on the CRF-induced ERK1/2 phosphorylation,

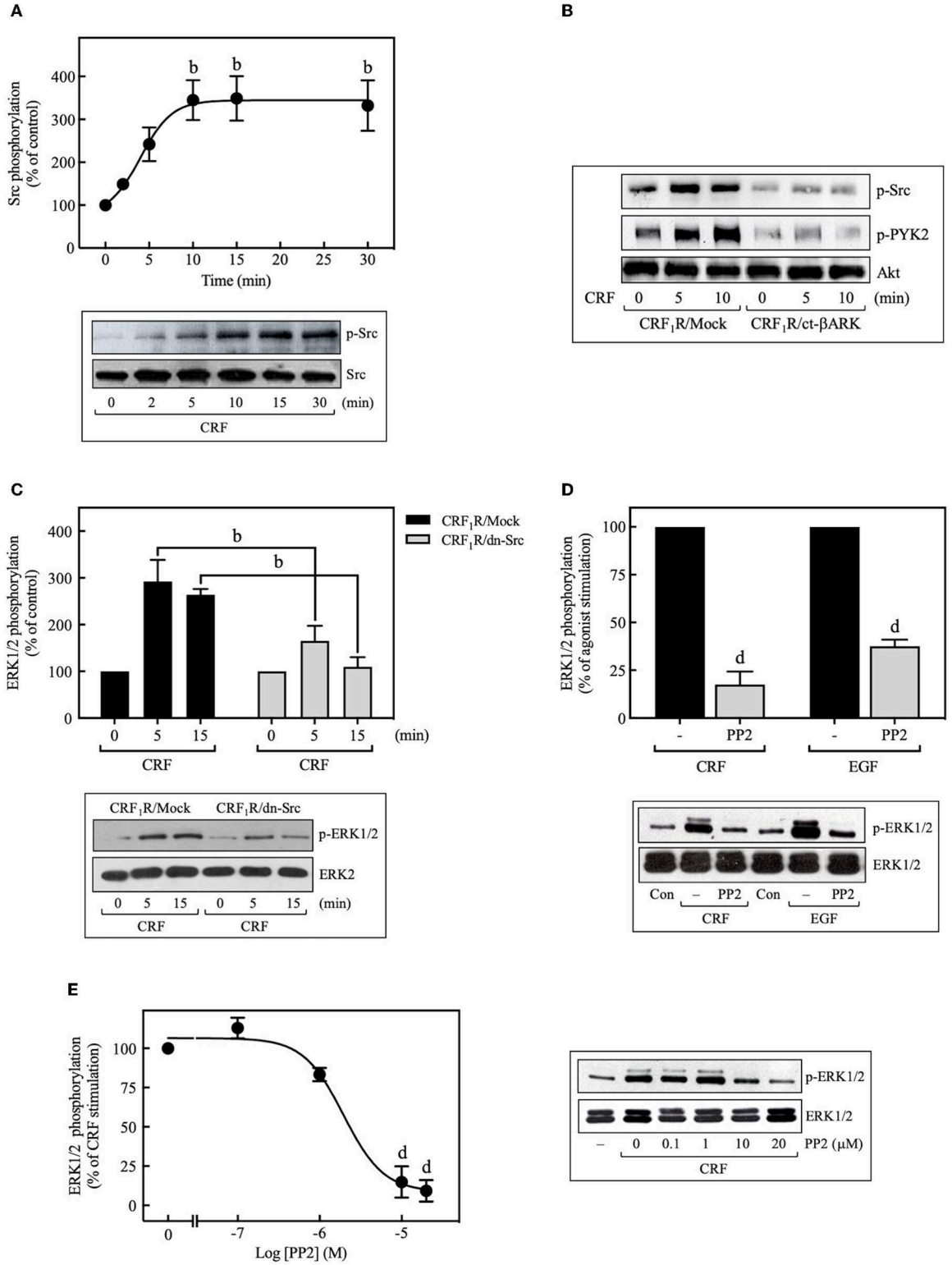

FIGURE 5 | Role of Src tyrosine kinase in CRF-stimulated ERK1/2 phosphorylation. **(A)** COS-7 cells expressing HA-CRF_1Rs were stimulated with 100 nM CRF for the indicated times. **(B)** COS-7 cells co-transfected with a plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with 100 nM CRF for 5 or 10 min. **(C)** COS-7 cells co-transfected with a dominant-negative SrcYF-KM (dn-Src) expression vector or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with

(Continued)

FIGURE 5 | 100 nM CRF for 5 or 15 min. Cells were pretreated with 10 μM or the indicated concentrations of PP2 for 30 min before stimulation with 100 nM CRF (5 min) **(D,E)** or 10 ng/ml EGF (10 min) **(D)**. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Src Tyr^{416} **(A,B)** or anti-p-ERK1/2 Thr^{202}/Tyr^{204} **(C–E)**, as described in Materials and Methods. Src, PYK2, and ERK1/2 phosphorylation were quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total Src, total Akt, and total ERK showing equal loading. **(A)** $^{b}p < 0.01$ vs. 0 min. **(C)** $^{b}p < 0.01$ vs. CRF_1R/Mock (5 or 15 min). **(D)** $^{d}p < 0.0001$ vs. CRF (–), $^{d}p < 0.0001$ vs. EGF (–). **(E)** $^{d}p < 0.0001$ vs. 0 M.

suggesting the participation of G_i protein and Gβγ subunits in this process. It is important to note that none of the observed effects of PTX and ct-βARK on CRF actions were present on EGF-stimulated ERK1/2 and Akt phosphorylation (**Supplementary Figure S7**).

Pretreatment with selective PI3K inhibitors, wortmannin (100 nM) (**Figure 8A**), or LY294002 (10 μM) (**Figure 9C**) abolished CRF_1R-mediated Akt signaling activation. Similarly, inhibition of PI3K by 100 nM wortmannin abolished the stimulatory action of EGF on Akt (**Figure 8A**), thereby demonstrating that the PI3K pathway is required for both CRF- and EGF-induced Akt phosphorylation. Considering that an upstream PI3K mechanism can also regulate CRF_1R and CRF_2R signaling via the ERK1/2 cascade in A7r5, CATH.a, and transfected CHO cells (9, 12), we investigated the potential role of PI3K in the activation of ERK1/2 by HA-CRF_1Rs expressed in COS-7 cells. In this context, activation of RTKs, such as the EGFRs, has been shown to recruit PI3K and activate ERK1/2 (50–53). However, contradictory data on PI3K involvement in EGFR-induced ERK1/2 phosphorylation have been reported (54–56). In this regard, to find out if EGF-mediated ERK1/2 phosphorylation observed in COS-7 cells is depending on PI3K activation, we analyze the effect of 100 nM wortmannin on the EGF ERK1/2 activation. As shown in **Figure 8A**, pretreatment with wortmannin was unable to inhibit the effect of EGF, suggesting that PI3K does not participate in this mechanism. In contrast, pretreatment with wortmannin abolished CRF-stimulated ERK1/2 phosphorylation (**Figure 8A**) in a concentration-dependent manner (0–100 nM), confirming an intermediary role for PI3K in CRF_1R ERK signaling (**Figure 8B**). To examine the contribution of CRF-mediated activation of Akt to the phosphorylation of ERK1/2, we evaluated the effect of the dn-Akt mutant. As shown in **Figure 8C**, overexpression of dn-Akt had no significant effect on ERK1/2 activation after stimulation with CRF (**Figure 8C**), suggesting that Akt does not participate in the activation of ERK1/2 by CRF. Because in the present work we do not show evidence about impairment of kinase activity of the dn-Akt, it will be necessary the use of other approaches, such as genetic tools or inhibitors, to provide more evidence regarding the possible no effect of Akt on the ERK 1/2 pathway. Because in the present work we do not show evidence about impairment of kinase activity of the dn-Akt, it will be necessary the use of other approaches, such as genetic tools or inhibitors, to provide more evidence regarding the possible Akt lack of effect on ERK 1/2 pathway. Consequently, our results suggest that PI3K can regulate the transduction of CRF_1R signals through the ERK cascade, possibly independently of Akt.

Src Acts Upstream and PI3K Downstream of the EGFR During CRF-Induced ERK1/2 Activation

Since we found that CRF-induced EGFR transactivation mediates ERK1/2 phosphorylation through Src- and PI3K-dependent mechanisms, we next determined if CRF-induced PI3K activation occurs upstream or downstream of Src and EGFR. Wortmannin inhibition of PI3K had no effect on CRF-induced phosphorylation of EGFR at Tyr^{845} (**Figure 9A**), suggesting that PI3K acts downstream of Src and EGFR. Consistent with these results, we also observed that wortmannin pretreatment did not alter CRF-induced phosphorylation of Src at Tyr^{416} (**Figure 9B**). Therefore, CRF_1R-stimulated transactivation of EGFR and phosphorylation of ERK1/2 mediated by Src was independent of PI3K. It has been reported that the PI3K/Akt signaling pathway can be activated at least by two independent mechanisms: (i) EGFR transactivation (57), and (ii) upstream Src activation (58, 59). We observed that CRF-induced Akt activation was completely inhibited by the Src inhibitor, PP2 (**Figure 9C**), suggesting that Src is an upstream regulator of PI3K and Akt. We next measured the effect of the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478, on CRF-stimulated Akt phosphorylation. As observed in **Figure 9D**, while CRF-induced ERK1/2 activation was totally dependent on EGFR transactivation (**Figure 9D**), Akt phosphorylation was only partially dependent. Thus, we hypothesize that PI3K/Akt pathway signaling by CRF_1R may involve two mechanisms: (i) a strong dependence on upstream Src activating PI3K and then Akt (**Figure 9C**); (ii) a weak dependence on EGFR transactivation (**Figure 9D**).

Role of β-Arrestin-2 in the CRF-Mediated ERK1/2 Activation

In recent years, it has been identified that β-arrestin proteins play an important role in mediating the actions of GPCRs, particularly those related to activation and regulation of Src and mitogenic pathways, in particular, the ERK1/2 signaling cascade (60). To determine the role of β-arrestins in the CRF-mediated ERK1/2 activation observed above, we used a phosphorylation-deficient mutant CRF_1R, which also shows a diminished agonist-dependent β-arrestin-2 recruitment (24). As shown in **Figure 10A**, COS-7 cells transiently transfected with HA-CRF_1R-Δ386 mutant showed a similar response in ERK1/2 phosphorylation compared to that observed with CRF_1R. The apparent independence of CRF-mediated activation of ERK1/2 from β-arrestin-2 could be explained by the low β-arrestin expression level

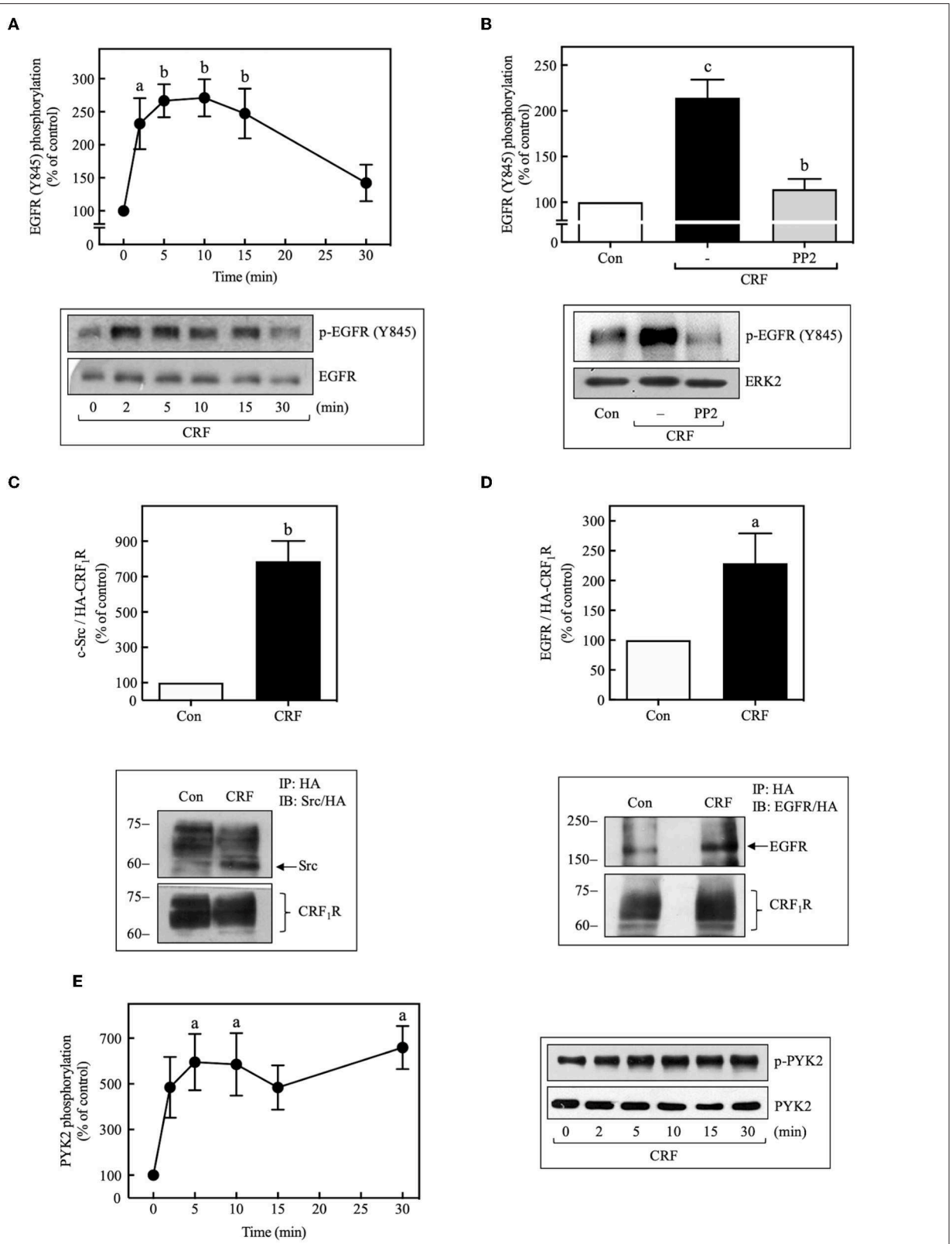

FIGURE 6 | CRF mediates EGFR phosphorylation at Tyr^{845} and activation of PYK2. COS-7 cells expressing HA-CRF_1Rs were stimulated with 100 nM CRF for the indicated times **(A,E)** or pretreated with 10 μM PP2 for 30 min before stimulation with 100 nM CRF for 5 min **(B)**. Total cell lysates were separated by SDS-PAGE

(Continued)

FIGURE 6 | and analyzed by immunoblotting with anti-p-EGFR Tyr^{845} **(A,B)** or anti-p-PYK2 Tyr^{402} **(E)**, as described in Materials and Methods. EGFR and ERK1/2 phosphorylation were quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total EGFR, ERK, or PYK2 showing equal loading. **(A)** $^{a}p < 0.05$, $^{b}p < 0.01$ vs. 0 min. **(B)** $^{c}p < 0.001$ vs. Con, $^{b}p < 0.01$ vs. CRF (–). **(E)** $^{a}p < 0.05$ vs. 0 min. **(D)** COS-7 cells expressing HA-CRF_1Rs were stimulated with 100 nM CRF for 10 min. Total cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-EGFR antibody or anti-HA antibody. **(C)** Blots were also stripped and reprobed with anti-Src polyclonal antibody. Src and EGFR were quantitated by densitometry, and mean values were plotted from three independent experiments. Vertical lines represent the S.E.M. **(C)** $^{b}p < 0.01$ vs. Con. **(D)** $^{a}p < 0.05$ vs. Con.

previously detected in COS-7 cells (61, 62). To assess this possibility, we evaluated the effect of β-arrestin-2 overexpression in COS-7 cells, since CRF_1R activation has been shown to lead to selective recruitment of β-arrestin-2 in both HEK293 cells and neurons (24, 63). As observed in **Figure 10B**, cells co-expressing HA-CRF_1R and β-arrestin-2 showed a significant increase in the CRF-mediated ERK1/2 phosphorylation, suggesting that β-arrestin involvement in CRF_1R ERK1/2 signaling depends on its cellular expression levels.

DISCUSSION

In the present study, we investigated the molecular mechanisms associated with the activation of ERK1/2 and Akt signaling cascades by the human CRF_1R in COS-7 cells. Our data suggest that agonist-stimulated CRF_1R promotes G_i activation and Gβγ release which, in turn, stimulate phosphorylation and activation of Src kinase. Once Src is active, it mediates ERK1/2 phosphorylation by at least two independent signaling mechanisms: (i) phosphorylation and transactivation of the EGFR, (ii) activation of PI3K. Interestingly, CRF_1R-induced Akt phosphorylation also requires Src-mediated activation of PI3K as the main mechanism, but it is mostly independent of EGFR transactivation.

Defining the molecular mechanisms for ERK1/2 signaling by a GPCR has become a significant focus of signal transduction research due to the multifaceted pathways mediating signaling via the ERK1/2-MAP kinase cascade. A significant role of the ERK1/2-MAP kinase pathway has been recognized in the biological action of both CRF_1R and CRF_2R. ERK1/2 is widely distributed in the brain and is considered an essential regulator of the molecular processes involved in response to stress (6, 64). It is well-established that most GPCRs signal *via* ERK1/2-MAP kinase cascades through distinct G_i-, G_s-, and G_q-dependent signaling pathways. In the case of the CRF_1R, it has been identified that the G_s/PKA pathway is importantly involved in the activation of MAP kinase cascades (12, 15, 18). In contrast, we found that pretreating CRF_1R-expressing COS-7 cells with PKA inhibitors H89 or Rp-cAMP did not alter the ability of CRF to stimulate ERK1/2 phosphorylation. Although earlier research proposed that high cellular expression of the serine-threonine kinase B-Raf molecularly switches "upstream" ERK1/2 activation by G_s-coupled GPCRs to a PKA mechanism (14), pretreating fetal hippocampal cells with the PKA inhibitor H89 only produced a small reduction in CRF_1R-mediated ERK phosphorylation despite very high hippocampal levels of B-Raf (18). Furthermore, H89 failed to inhibit CRF_1R-mediated ERK signaling in brain-derived CATH.a, rat fetal microglial, locus coeruleus, and transfected CHO cells (8, 9, 12, 17). In fact, ERK activation by CRF_1R in HEK293 cells was markedly decreased after the third intracellular loop's Ser^{301} was phosphorylated by PKA (65). Thus, a cAMP-dependent PKA → Rap1 → B-Raf mechanism does not always mediate ERK1/2 signaling by G_s-coupled receptors. EPAC, a guanine nucleotide exchange factor that is activated by intracellular cAMP, has been shown to regulate activation of Rap1 and ERK1/2 without the involvement of PKA (66). G_s-coupled CRF_1R signaling can stimulate ERK1/2 phosphorylation by activating upstream EPAC2 independent of PKA in certain cell lines (17, 67). Interestingly, neither Epac nor PKA was found to mediate Akt cascade signaling by $CRF_{2(b)}R$ in HEK293 (21).

The versatility of the CRF_1R to activate different signaling pathways has allowed its coupling to G_q proteins to be identified (4). G_q conveys a signal to activate PKC which then triggers MAP kinase cascades. Thus, it has been shown that G_q/PLC/PKC cascade signaling by CRF_1R activated by Ucn1 contributes to phosphorylation of ERK1/2 in CRF_1R-expressing myometrial, CHO, HEK293, and rat hippocampal cells (12, 13, 18). However, in pituitary AtT20 cells and CATH.a cells, PKC is not involved in Ucn1-stimulated ERK1/2 phosphorylation (12). In our study, pretreatment with the PKC inhibitor, BIM, increased rather than reduced CRF-stimulated ERK1/2 phosphorylation, suggesting that PKC may negatively regulate CRF_1R ERK1/2 signaling in COS-7 cells, although the specific mechanism for this effect remains to be determined.

The use of PTX in our study suggests the participation of G_i protein in the CRF-dependent activation of ERK and Akt pathways. Interestingly, it is now well-established that during GPCR/G_i signaling, Gβγ release can activate a myriad of effectors to modulate diverse signaling pathways downstream of GPCRs, including Src, which in turn activate EGFR to promote ERK1/2 activation (43, 68, 69). Gβγ-activated Src can also associate PYK2. When we blocked that action of Gβγ subunits in COS-7 cells by overexpressing the ct-βARK peptide, which is a Gβγ subunit scavenger (70, 71), CRF-stimulated ERK phosphorylation was decreased by ∼40%. Moreover, ct-βARK overexpression markedly reduced phosphorylation of Src and Akt. In agreement, another group has also found that CRF_1R ERK1/2 signaling is only partially dependent on Gβγ, although their study did not assess the role of Gβγ subunits in the activation of upstream ERK1/2 pathways. Differences in CRF_1R-mediated activation of the ERK1/2-MAP kinase cascade are probably attributable to variations

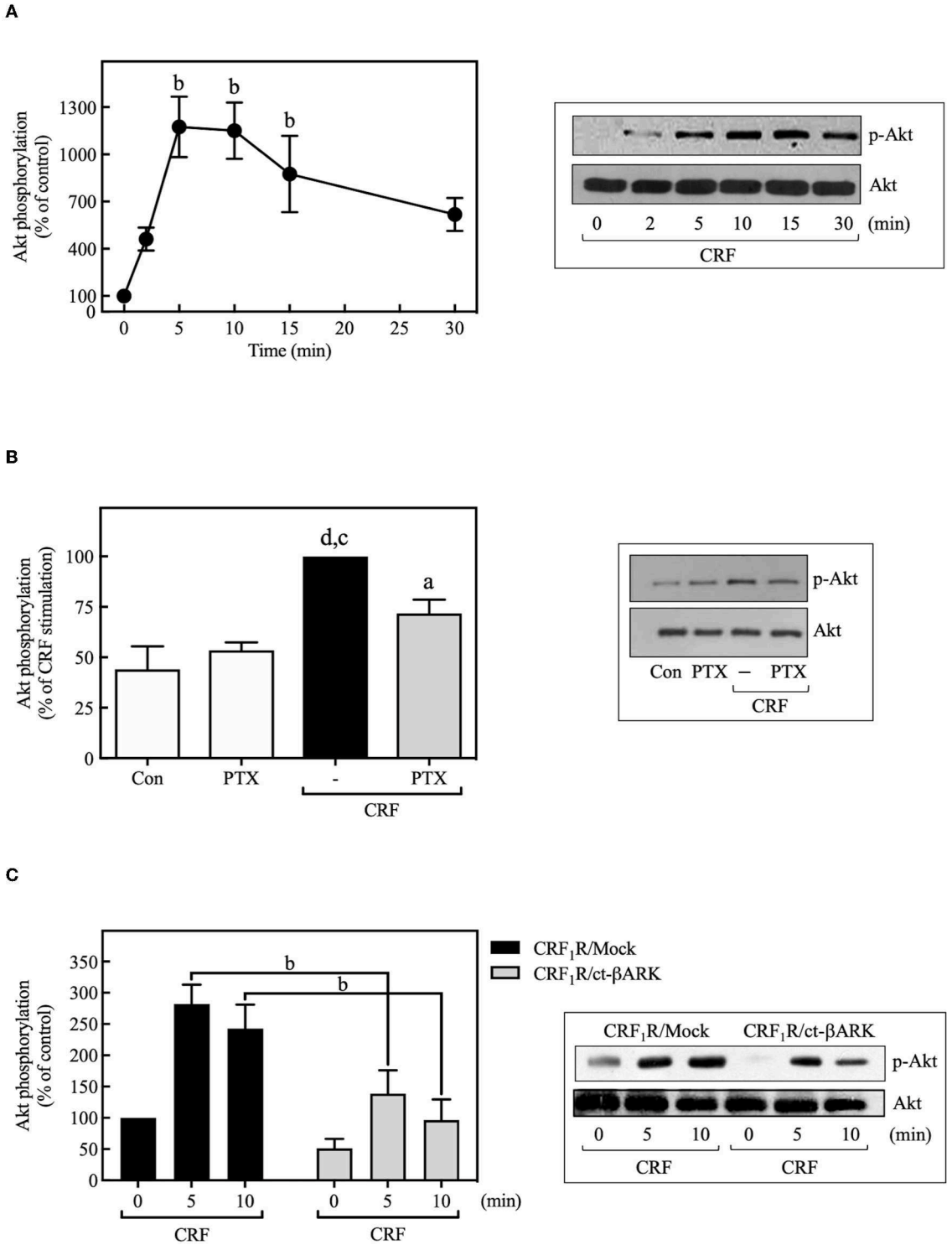

FIGURE 7 | CRF mediates Akt activation. **(A)** COS-7 cells expressing HA-CRF_1Rs were stimulated with 100 nM CRF for the indicated times. **(B)** COS-7 cells expressing HA-CRF_1Rs were pretreated with 100 ng/ml PTX for 15 h before stimulation with 100 nM CRF for 5 min. **(C)** COS-7 cells co-transfected with a plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with 100 nM CRF for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt Ser^{473}, as described in Materials and Methods. Akt phosphorylation was quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total Akt showing equal loading. **(A)** $^{b}p < 0.01$ vs. 0 min. **(B)** $^{d}p < 0.0001$ vs. Con, $^{c}p < 0.001$ vs. PTX; $^{a}p < 0.05$ vs. CRF (–). **(C)** $^{b}p < 0.01$ vs. CRF_1R/Mock (5 and 10 min).

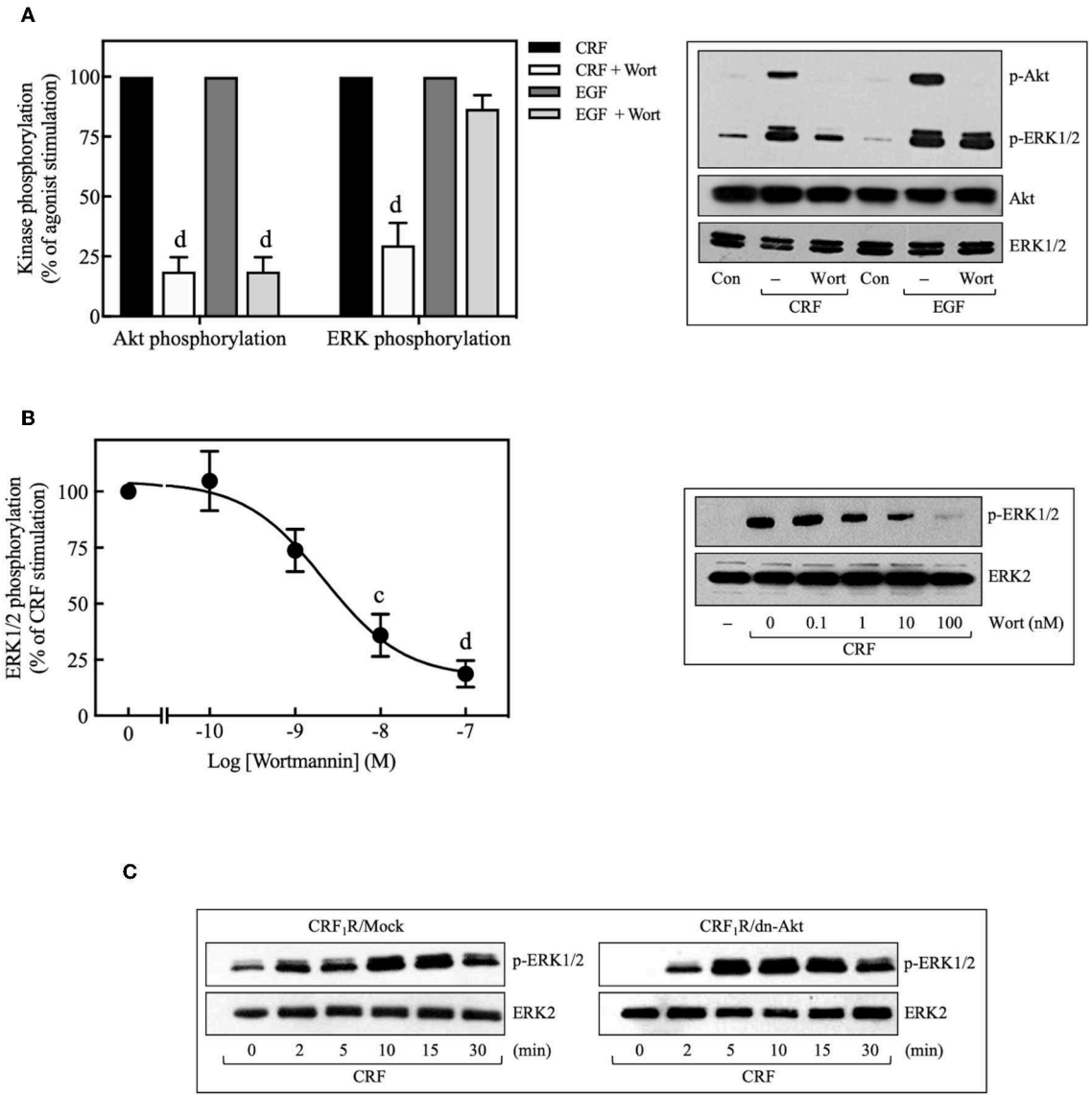

FIGURE 8 | Involvement of the PI3K pathway in CRF-stimulated ERK1/2 phosphorylation. **(A,B)** COS-7 cells expressing HA-CRF_1Rs were pretreated with 100 nM or the indicated concentrations of wortmannin (Wort) for 30 min before stimulation with 100 nM CRF for 5 min **(A,B)** or 10 ng/ml EGF for 10 min **(A)**. **(C)** COS-7 cells co-transfected with a dominant-negative Akt (K179M) expression vector (dn-Akt) or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with 100 nM CRF for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt Ser^{473} **(A)** or anti-p-ERK1/2 Thr^{202}/Tyr^{204} **(A–C)**, as described in Materials and Methods. Akt and ERK1/2 phosphorylation were quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total Akt or ERK showing equal loading. **(A)** $^{d}p < 0.0001$ vs. CRF (p-Akt), $^{d}p < 0.0001$ vs. EGF (p-Akt); $^{d}p < 0.0001$ vs. CRF (p-ERK), **(B)** $^{c}p < 0.001$, $^{d}p < 0.0001$ vs. 0 M.

in the signaling properties of transfected CRF_1Rs expressed in different cell lines utilized in these studies. We are presently investigating other upstream factors including β-arrestins that regulate Src and EGFR mediation of CRF_1R ERK1/2 signaling.

Our experiments did demonstrate that CRF-stimulated phosphorylation of ERK1/2 and EGFR occurred in parallel, while pretreatment with the EGFR kinase inhibitor, AG1478, caused a concentration-dependent inhibition of CRF-stimulated ERK1/2 phosphorylation. In agreement, it has been shown that EGFR transactivation is required for Ucn1-stimulated ERK1/2 phosphorylation in transfected HEK293 cells (5). In contrast to our data indicating that a MMP/HB-EGF ligand mechanism was not involved, however, this group reported that MMP generation of an HB-EGF ligand transactivated the EGFR during CRF_1R ERK1/2 signaling (5). Therefore, EGFR transactivation can play a critical role in CRF_1R signaling via the ERK1/2-MAP kinase cascade.

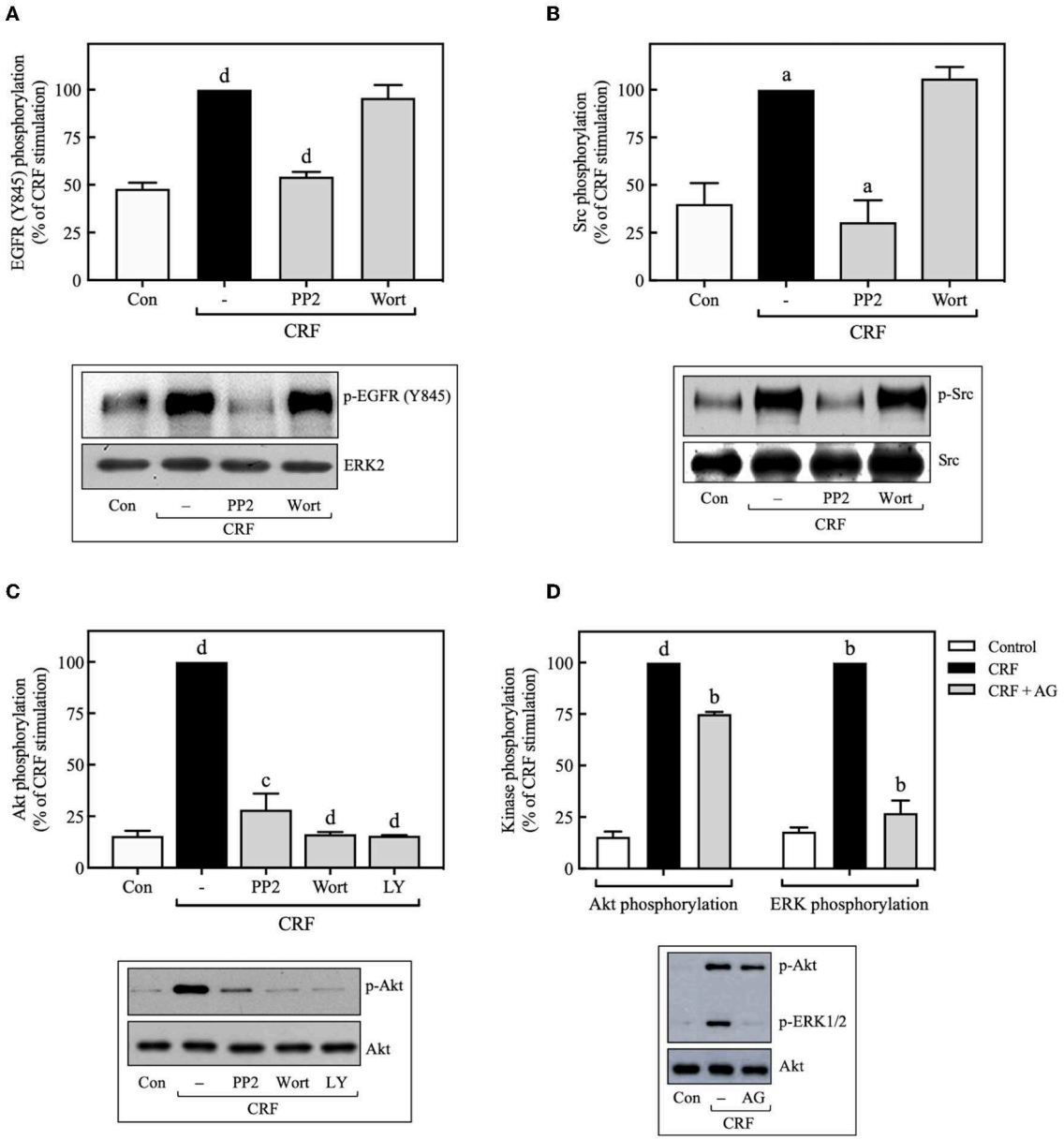

FIGURE 9 | Src is an upstream regulator of EGFR and PI3K. COS-7 cells expressing HA-CRF_1Rs were pretreated with 10 μM PP2, 100 nM wortmannin (Wort) **(A–C)** or 10 μM LY294002 (LY) **(C)** for 30 min, before stimulation with 100 nM CRF for 5 min. **(D)** Effect of 100 nM AG1478 (AG) on CRF- (100 nM, 5 min) induced ERK1/2 and Akt phosphorylation. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-EGFR Tyr^{845} **(A)**, anti-p-Src Tyr^{416} **(B)**, anti-p-Akt Ser^{473} **(C,D)** or anti-p-ERK1/2 Thr^{202}/Tyr^{204} **(D)**, as described in Materials and Methods. EGFR, Src, Akt, and ERK1/2 phosphorylation were quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total ERK, Src or Akt showing equal loading. **(A)** $^{d}p < 0.0001$ vs. Con; $^{d}p < 0.0001$ vs. CRF (–). **(B)** $^{a}p < 0.05$ vs. Con; $^{a}p < 0.05$ CRF(–). **(C)** $^{d}p < 0.0001$ vs. Con; $^{c}p < 0.001$, $^{d}p < 0.0001$ vs. CRF (–). **(D)** $^{d}p < 0.0001$ vs. Con (p-Akt), $^{b}p < 0.01$ vs. CRF (p-Akt); $^{b}p < 0.01$ vs. Con (p-ERK), $^{b}p < 0.01$ vs. CRF (p-ERK).

Earlier studies have implicated a PI3K-dependent mechanism in CRF_1R ERK1/2 signaling based on the observation that pretreatment with PI3K inhibitors attenuated sauvagine- and Ucn1-stimulated ERK1/2 phosphorylation in CRF_1R-expressing CHO and HEK293 cells (5, 9, 12). PI3K is also involved in $CRF_{2(b)}R$-stimulated ERK1/2 activation in CHO, A7r5, and mouse neonatal cardiomyocyte cells (12, 19). However, the activation sequence of PI3K, EGFR, and ERK1/2 during CRF_1R signaling has not been fully elucidated. Here we observed that pretreating CRF_1R-expressing COS-7 cells with the PI3K inhibitors wortmannin and LY294002 inhibited CRF-stimulated phosphorylation of ERK1/2 and Akt. Previous studies suggest that PI3K activity is required for Gβγ-mediated MAP kinase signaling pathway at a point upstream of Sos and Ras activation (50, 72).

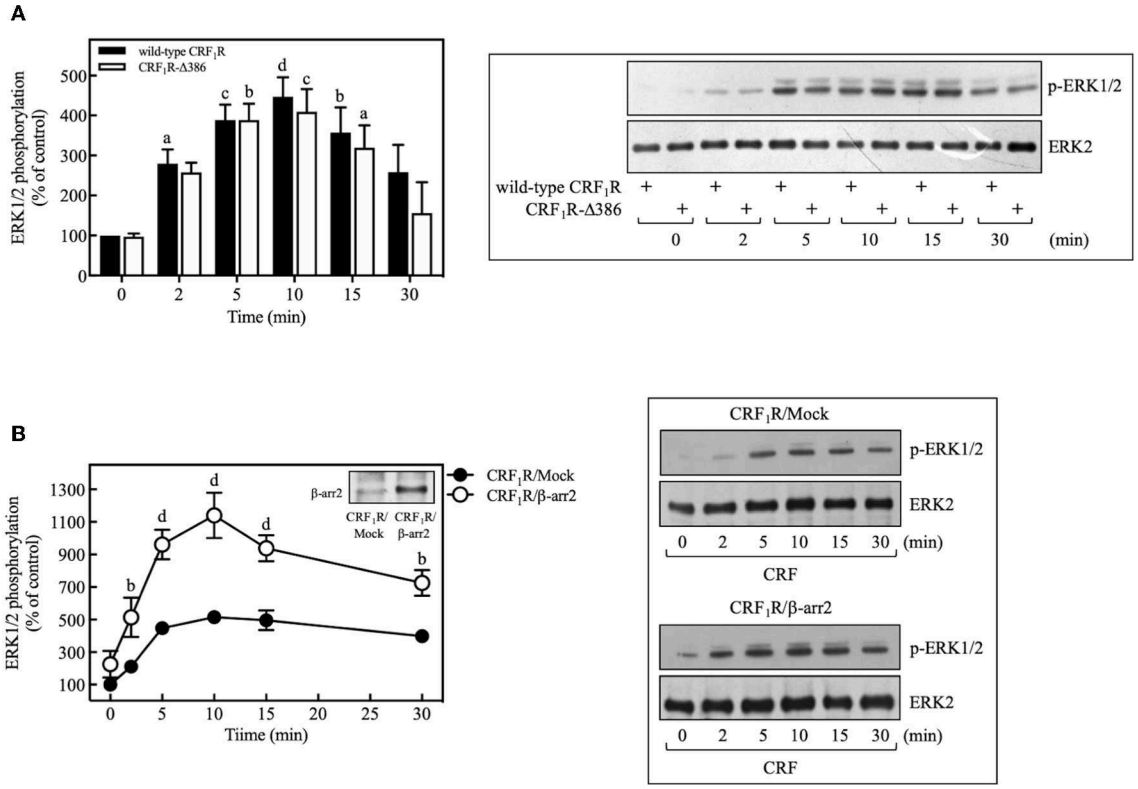

FIGURE 10 | β-arrestin does not participate in the CRF-stimulated ERK 1/2 phosphorylation in COS-7 cells. **(A)** COS-7 cells expressing HA-CRF_1R (wild-type CRF_1R) or HA-CRF_1R-Δ386 mutant were stimulated with 100 nM CRF for the indicated times. **(B)** COS-7 cells co-transfected with a β-arrestin-2 (β-arr2) expression vector or an empty control vector (Mock) and the pcDNA3-HA-CRF_1R expression vector were stimulated with 100 nM CRF for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-ERK1/2 Thr^{202}/Tyr^{204}, as described in Materials and Methods. ERK1/2 phosphorylation was quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total ERK showing equal loading. **(A)** $^{a}p < 0.05$, $^{c}p < 0.001$, $^{d}p < 0.0001$, $^{b}p < 0.01$ vs. 0 min (CRF_1R); $^{b}p < 0.01$, $^{c}p < 0.001$, $^{a}p < 0.05$ vs. 0 min (CRF_1R-Δ386). **(B)** $^{b}p < 0.01$ vs. CRF_1R (2 or 30 min); $^{d}p < 0.0001$ vs. CRF_1R (5, 10, or 15 min).

Because we also found that AG1478 abolished phosphorylation of ERK1/2 while only decreasing Akt phosphorylation 25% in transfected COS-7 cells stimulated with CRF, upstream activation of the PI3K/Akt pathway by CRF_1R is not strongly dependent on EGFR transactivation. In this context, our study suggests that Src acts as a critical mediator of PI3K activation, independent of EGFR transactivation, which, in turn, stimulates Akt and ERK1/2 phosphorylation. Previous studies have shown that activated Src directly associates with PI3K through interaction between the SH3 domain of Src and the proline-rich motif in the p85 regulatory subunit of PI3K, thereby increasing the specific activity of PI3K (59). Furthermore, intermediary proteins have also been identified to mediate Src-induced PI3K activation, such as p66Shc, Rap1, and FAK. Thus, our study raises the possibility that Src activates PI3K, although the specific mechanism for this effect remains to be determined.

For certain GPCRs, Src has been shown to induce EGFR transactivation, stimulate the PI3K-Akt pathway, and activate the ERK1/2 cascade (43, 48, 70). A novel finding in our study is the rapid and parallel phosphorylation of Src, PYK2, the EGFR, Akt, and ERK1/2 in CRF_1R-expressing COS-7 cells stimulated with CRF. Importantly, we demonstrated that inhibiting Src function with PP2 markedly reduced or abolished the CRF-stimulated activation of Src, PYK2, the EGFR, and ERK1/2, suggesting that Src has a central role in regulating CRF_1R ERK1/2 signaling. Thus, our results clearly show that Src triggers signal transduction by two important pathways culminating in ERK activation by CRF_1R: (i) EGFR activation of the classical Ras/Raf/MEK/ERK pathway, and (ii) PI3K regulation and subsequent activation of ERK1/2 (**Figure 11**).

To the best of our knowledge, our study demonstrates for the first time that Src regulates ERK and Akt signaling by the CRF_1R. Yuan et al. (20) reported that Src was an upstream regulator of ERK signaling by both the CRF_1R and CRF_2R in the mouse atrial HL-1 cardiomyocytes cell line based on the effects of antalarmin (a CRF_1R antagonist) and anti-sauvagine (a CRF_2R antagonist). Although CRF_1R was reported to be expressed in the human heart (73, 74), Ikeda et al. (75) reported that $CRF_{2(b)}R$ is the major CRF receptor expressed in the HL-1 mouse atrial cardiomyocyte cell line with no measurable level of CRF_1R mRNA. Their data detecting only

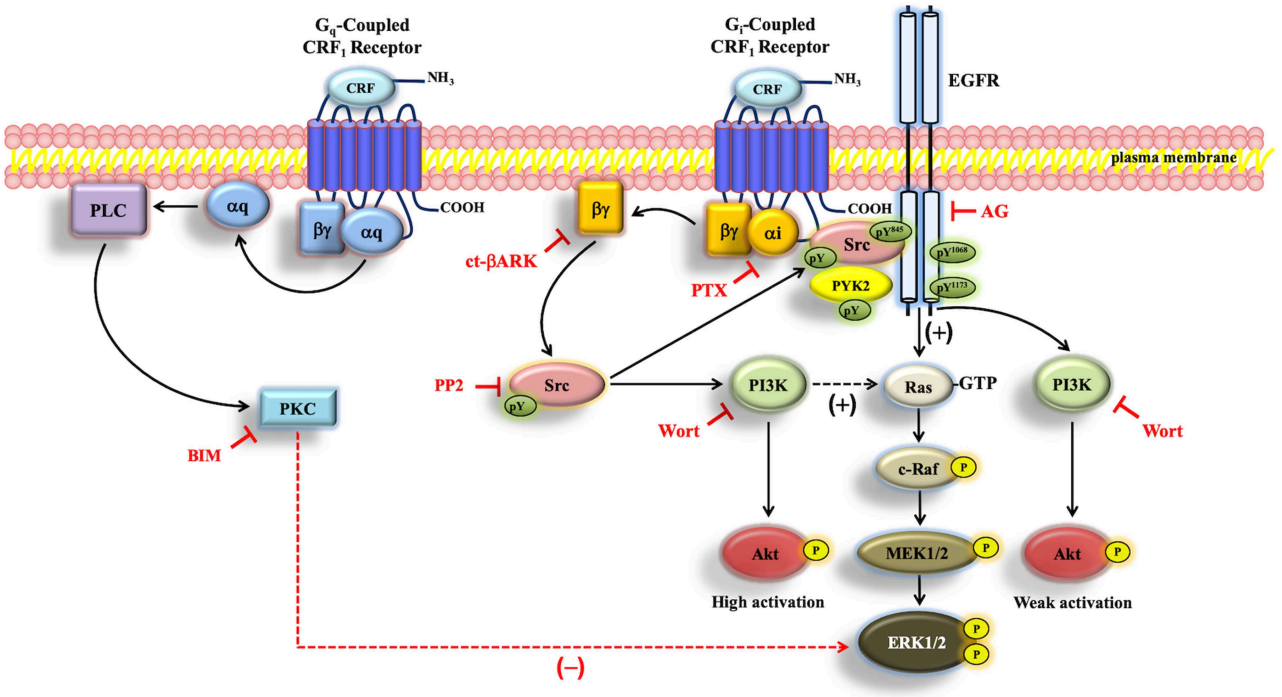

FIGURE 11 | Schematic representation of the proposed CRF-mediated ERK1/2/Akt signaling mechanism in CRF_1R transfected COS-7 cells. When COS-7 cells are stimulated with CRF, the overexpressed CRF_1R leads to activation of G_i protein and the subsequent dissociation of α_i-GTP and βγ. Gβγ subunit is able to activate Src, which plays a central role in the activation of EGFR, through the formation of a protein complex that contains CRF_1R, EGFR, and Src. PI3K, independently of Akt, is involved in ERK1/2 activation, presumably through Ras/C-Raf/MEK1/2. On the other hand, Src/PYK2 transactivates EGFR. Such EGFR transactivation leads to activation of the MAP kinase/ERK1/2 cascade and parallelly to weak activation of the PI3K/Akt pathway. Solid arrows indicate signaling mechanisms that have been identified. Dashed arrows indicate that the precise mechanism associated with the CRF-mediated regulation of Ras by PI3K and ERK1/2 by PKC remains to be determined. Plus sign (+) means positive regulation, and minus sign (–) means negative regulation of the ERK pathway.

CRF_2R expression in HL-1 cells is consistent with previous and more recent studies demonstrating only CRF_2R expression in rat and mouse cardiomyocytes (19, 76, 77). Therefore, ERK1/2 signaling stimulated by Ucns in cardiomyocytes is mediated through CRF_2R, which appears to be the main mediator of the cardiac stress response (78, 79), rather than through CRF_1R. Additionally, recent observations also indicate that CRF_2R controls the cellular organization and colon cancer progression, specifically through the Src/ERK pathway (80, 81). Thus, while all previous findings are relevant to CRF_2Rs, our findings show for the first time that Src plays an important role in the regulation of ERK1/2 and Akt signaling by the CRF_1R.

An important finding of our study was the detection of a signaling protein scaffold, which contains CRF_1R, Src, and EGFR (**Figures 6C,D**). While the association between CRF_1R and Src was totally dependent on CRF agonist activation, a constitutive interaction between CRF_1R and EGFR was also detected, which was increased after CRF stimulation. In this context, it has previously reported that some GPCRs physically interact with EGFR in the absence of receptor ligands, a condition that may increase the efficiency of EGFR transactivation (29, 82–84). Thus, it is possible that the detected constitutive association between CRF_1R and EGFR facilitates a more rapid CRF agonist-induced recruitment of Src to the EGFR and subsequent phosphorylation and activation of EGFR. With regard to this possibility, the presence of a putative proline-rich domain-binding SH3 motif (*ProXXPro;* X, any amino acid), located in the carboxyl terminus of the CRF_1R ($Pro^{398}Thr^{399}Ser^{400}Pro^{401}$) may provide a site for the direct interaction between Src and CRF_1R after agonist stimulation. However, the CRF_1R-Δ386 mutant, which lacks the *ProXXPro* motif, induces a similar degree of ERK1/2 activation that is induced by the wild-type CRF_1R, which suggests this putative region may not participate in the binding to Src (**Figure 10A**).

Moreover, there is evidence that Tyr phosphorylation of GPCRs plays a role in mediating GPCR-Src interactions (43). For instance, in studies conducted in A431 epidermoid carcinoma cells, stimulation of the β_2-adrenergic receptor (β_2-AR) with isoproterenol, results in phosphorylation of the receptor on Tyr^{305} (43, 85). The mutation of this residue to Phe abolishes Src/β_2-AR association and impairs Src activation. This residue lies within a canonical Src SH2 binding domain, and it is proposed that Src directly binds the Tyr-phosphorylated β_2-AR. Interestingly, the CRF_1R has also a single putative SH2 binding domain (*TyrXX-hyd*; hyd, hydrophobic amino acid) located at the end of the third intracellular loop ($Tyr^{309}Arg^{310}Lys^{311}Ala^{312}$),

which may be a site where Src can directly interact with CRF_1R. Further work is needed to establish the importance of this putative site in the agonist-induced CRF_1R/Src interaction and Src activation.

β-arrestins are a small family of cytosolic proteins initially identified for their central role in GPCRs desensitization. Furthermore, β-arrestins act as adaptors in clathrin-mediated receptor endocytosis (86). In this sense, their role in CRF_1R homologous desensitization and endocytosis is well-recognized, particularly for β-arrestin-2 (24, 63, 87). It is now well-established, however, that β-arrestins can also act as GPCR-signaling transducers that recruit and activate many other signaling molecules, including Src, MAP kinase, NF-κB and PI3K that modulate diverse cellular responses (64, 86). β-arrestin regulation of CRF/CRF_1R signaling is still not fully understood.

Regarding β-arrestin regulation of CRF/CRF_1R-mediated ERK1/2 activation, β-arrestin-2-mediation of CRF_1R internalization participates in the late phase of sustained ERK1/2 activation after G protein activation and B-Raf mediate the early phase of ERK1/2 activation (88). However, overexpression of PDS-95 in HEK293 cells, a CRF_1R-interacting protein, inhibited CRF-induced-CRF_1R internalization in a PDZ-binding motif-dependent manner by suppressing β-arrestin-2 recruitment. Intriguingly, neither the overexpression of PSD-95 nor the knockdown of endogenous PSD-95 affected CRF-mediated activation of ERK1/2 (89).

Under this experimental evidence and due to the importance of β-arrestins in the scaffolding and activation of Src and regulation of MAP kinase cascades, it was decided to evaluate their role in the CRF/CRF_1R-mediated ERK1/2 activation observed in COS-7 cells. Using a phosphorylation-deficient mutant CRF_1R, which has a decreased interaction with β-arrestin-2 (24), no significant changes in the activation of ERK1/2 were detected after agonist stimulation (**Figure 10A**), suggesting that β-arrestin-2 is not involved in the CRF/CRF_1R-mediated ERK1/2 activation observed in COS-7. This finding, however, can be explained in part by the low expression level of β-arrestins in COS-7 cells (61, 62). This hypothesis is supported by our data showing that overexpressing β-arrestin-2 in COS-7 notably increased the CRF/CRF_1R-mediated ERK1/2 activation (**Figure 10B**). Likewise, β-arrestin overexpression in COS-7 cells has been found to augment CRF_1R internalization (24). Thus, our data provide evidence about the involvement of β-arrestin-2 in the CRF/CRF_1R MAP kinase activation in cells with sufficient β-arrestin expression.

Our findings on signaling pathways activated by CRF_1R help to elucidate the molecular mechanisms involved in response to stress mediated by this receptor. For instance, kinases in the ERK1/2-MAP kinase cascade, including Src and PYK2, are highly expressed in extended amygdala and forebrain neurons regulating anxiety defensive behavior and stress responsiveness (90–92). Acute stress or central CRF administration induces rapid phosphorylation of ERK1/2 in the basolateral amygdala and hippocampal neurons and prominent anxiety-like behavior in rats and mice (93–95). Furthermore, CRF_1Rs can also signal through other cellular pathways that may be involved in post-traumatic stress disorder pathophysiology. As we showed here, CRF_1R activated by CRF stimulated rapid phosphorylation of Akt at Ser^{473} that is mediated by upstream Src and PI3K. Preclinical research has shown that activated Akt in the ventral tegmentum promotes resilience to anxiety- and depressive-like responses to stress (3, 96), while high levels of phosphorylated Akt in the dorsal hippocampus and basolateral amygdala prolongs contextual and sensitized fear induced by inescapable stress (3, 97). Therefore, the consequences of CRF_1R Akt signaling during trauma and severe stress may differ depending on the brain region. Hence, ERK1/2-MAP kinase and Akt cascade signaling by CRF_1R regulated by Src, PYK2, and EGFR may have critical roles in stress-induced anxiety and depression.

CONCLUSIONS

In summary, the data presented herein establish that the tyrosine kinase Src serves as a central upstream regulator of ERK1/2-MAP kinase and Akt cascade signaling by the human CRF_1R in COS-7 cells. Although CRF_1R coupling to G proteins strongly activates PKA and PKC pathways, neither second messenger kinases were involved in CRF_1R-mediated ERK1/2 signaling. However, Gβγ released during activation of CRF_1R by CRF, particularly from G_i, stimulates phosphorylation of Src and PYK2, which in turn promotes transactivation of the EGFR through the formation of a heterotrimeric complex formed by the association of CRF_1R, Src, and EGFR. EGFR transactivation, which occurred independent of MMP generation of the HB-EGF ligand, was essential for CRF-stimulated ERK1/2 phosphorylation while having only a small role in CRF_1R-mediated Akt activation. Although PI3K activation contributes to CRF-stimulated ERK1/2 phosphorylation, CRF_1R-mediated EGFR transactivation is independent of the PI3K/Akt pathway. In contrast, CRF_1R Akt signaling while also being mediated by generation of Gβγ and phosphorylation of Src is weakly dependent on EGFR transactivation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JO-R, RH, and KC conceived the project. JO-R, FD, and RH designed the experiments. GP-M, AF-G, JH-A, and MD-C carried out the experiments. JO-R, GP-M, and AF-G analyzed and discussed the data. JO-R and RH wrote the manuscript. All authors read and approved the final version of the manuscript and took a due care to ensure the integrity of the work.

FUNDING

JO-R was supported by the CINVESTAV-IPN, a University of California MEXUS-CONACYT grant for collaborative

projects, CONACYT grants (48777 and 167673) and a Grant for Research on Health from Fundacion Miguel Aleman-2018. RH was funded by the VA Center of Excellence for Stress and Mental Health (CESMH); a VA Merit Review grant, NIH RO1 grants AG050595 and AG022381 from the National Institute of Aging; and a University of California MEXUS-CONACYT grant for collaborative projects. GP-M held a CONACYT graduated scholarship (296029).

DEDICATION

This work is dedicated to Dr. Kevin J. Catt, who was an extraordinary scientist, mentor, and friend who passed away on October 1, 2017.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. Walter Koch for the plasmid pRK5 expressing the carboxyl terminus (βγ-binding domain) of βARK and Silvio Gutkind for the pCEFL-SrcYF-KM which contains the inactive form of SrcYF (dominant-negative). The authors would also like to thank Dr. Jose Antonio Arias-Montaño and M.Sc. Huguet Landa-Galvan, Cinvestav-IPN, Mexico, for their critical comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00869/full#supplementary-material

REFERENCES

1. Deussing JM, Chen A. The corticotropin-releasing factor family: physiology of the stress response. *Physiol Rev*. (2018) 98:2225–86. doi: 10.1152/physrev.00042.2017
2. Hillhouse EW, Grammatopoulos DK. The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. *Endocr Rev*. (2006) 27:260–86. doi: 10.1210/er.2005-0034
3. Hauger RL, Risbrough V, Oakley RH, Olivares-Reyes JA, Dautzenberg FM. Role of CRF receptor signaling in stress vulnerability, anxiety, and depression. *Ann N Y Acad Sci*. (2009) 1179:120–43. doi: 10.1111/j.1749-6632.2009.05011.x
4. Dautzenberg FM, Gutknecht E, Van der Linden I, Olivares-Reyes JA, Durrenberger F, Hauger RL. Cell-type specific calcium signaling by corticotropin-releasing factor type 1 (CRF1) and 2a (CRF2(a)) receptors: phospholipase C-mediated responses in human embryonic kidney 293 but not SK-N-MC neuroblastoma cells. *Biochem Pharmacol*. (2004) 68:1833–44. doi: 10.1016/j.bcp.2004.07.013
5. Punn A, Levine MA, Grammatopoulos DK. Identification of signaling molecules mediating corticotropin-releasing hormone-R1alpha-mitogen-activated protein kinase (MAPK) interactions: the critical role of phosphatidylinositol 3-kinase in regulating ERK1/2 but not p38 MAPK activation. *Mol Endocrinol*. (2006) 20:3179–95. doi: 10.1210/me.2006-0255
6. Arzt E, Holsboer F. CRF signaling: molecular specificity for drug targeting in the CNS. *Trends Pharmacol Sci*. (2006) 27:531–8. doi: 10.1016/j.tips.2006.08.007
7. Dermitzaki E, Tsatsanis C, Gravanis A, Margioris AN. Corticotropin-releasing hormone induces Fas ligand production and apoptosis in PC12 cells via activation of p38 mitogen-activated protein kinase. *J Biol Chem*. (2002) 277:12280–7. doi: 10.1074/jbc.M111236200
8. Wang W, Ji P, Dow KE. Corticotropin-releasing hormone induces proliferation and TNF-alpha release in cultured rat microglia *via* MAP kinase signalling pathways. *J Neurochem*. (2003) 84:189–95. doi: 10.1046/j.1471-4159.2003.01544.x
9. Rossant CJ, Pinnock RD, Hughes J, Hall MD, McNulty S. Corticotropin-releasing factor type 1 and type 2alpha receptors regulate phosphorylation of calcium/cyclic adenosine 3′,5′-monophosphate response element-binding protein and activation of p42/p44 mitogen-activated protein kinase. *Endocrinology*. (1999) 140:1525–36. doi: 10.1210/endo.140.4.6656
10. Cao J, Cetrulo CL, Theoharides TC. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. *Mol Pharmacol*. (2006) 69:998–1006. doi: 10.1124/mol.105.019539
11. Park HJ, Kim HJ, Lee JH, Lee JY, Cho BK, Kang JS, et al. Corticotropin-releasing hormone (CRH) downregulates interleukin-18 expression in human HaCaT keratinocytes by activation of p38 mitogen-activated protein kinase (MAPK) pathway. *J Invest Dermatol*. (2005) 124:751–5. doi: 10.1111/j.0022-202X.2005.23656.x
12. Brar BK, Chen A, Perrin MH, Vale W. Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides. *Endocrinology*. (2004) 145:1718–29. doi: 10.1210/en.2003-1023
13. Grammatopoulos DK, Randeva HS, Levine MA, Katsanou ES, Hillhouse EW. Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated *via* R1alpha and R2beta CRH receptor subtypes and stimulation of Gq-proteins. *Mol Endocrinol*. (2000) 14:2076–91. doi: 10.1210/mend.14.12.0574
14. Stork PJS. Does Rap1 deserve a bad Rap? *Trends Biochem Sci*. (2003) 28:267–75. doi: 10.1016/S0968-0004(03)00087-2
15. Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, et al. Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. *Mol Endocrinol*. (2002) 16:1638–51. doi: 10.1210/mend.16.7.0863
16. Kageyama K, Hanada K, Moriyama T, Imaizumi T, Satoh K, Suda T. Differential regulation of CREB and ERK phosphorylation through corticotropin-releasing factor receptors type 1 and 2 in AtT-20 and A7r5 cells. *Mol Cell Endocrinol*. (2007) 263:90–102. doi: 10.1016/j.mce.2006.08.011
17. Traver S, Marien M, Martin E, Hirsch EC, Michel PP. The phenotypic differentiation of locus ceruleus noradrenergic neurons mediated by brain-derived neurotrophic factor is enhanced by corticotropin releasing factor through the activation of a cAMP-dependent signaling pathway. *Mol Pharmacol*. (2006) 70:30–40. doi: 10.1124/mol.106.022715
18. Pedersen WA, Wan R, Zhang P, Mattson MP. Urocortin, but not urocortin II, protects cultured hippocampal neurons from oxidative and excitotoxic cell death via corticotropin-releasing hormone receptor type I. *J Neurosci*. (2002) 22:404–12. doi: 10.1523/JNEUROSCI.22-02-00404.2002
19. Brar BK, Jonassen AK, Egorina EM, Chen A, Negro A, Perrin MH, et al. Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart. *Endocrinology*. (2004) 145:24–35; discussion 21-3. doi: 10.1210/en.2003-0689
20. Yuan Z, McCauley R, Chen-Scarabelli C, Abounit K, Stephanou A, Barry SP, et al. Activation of Src protein tyrosine kinase plays an essential role in urocortin-mediated cardioprotection. *Mol Cell Endocrinol*. (2010) 325:1–7. doi: 10.1016/j.mce.2010.04.013
21. Markovic D, Punn A, Lehnert H, Grammatopoulos DK. Molecular determinants and feedback circuits regulating type 2 CRH receptor signal integration. *Biochim Biophys Acta-Mol Cell Res*. (2011) 1813:896–907. doi: 10.1016/j.bbamcr.2011.02.005
22. Dautzenberg FM, Kilpatrick GJ, Wille S, Hauger RL. The ligand-selective domains of corticotropin-releasing factor type 1 and type 2 receptor

reside in different extracellular domains: generation of chimeric receptors with a novel ligand-selective profile. *J Neurochem*. (1999) 73:821–9. doi: 10.1046/j.1471-4159.1999.0730821.x

23. Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem*. (2000) 275:17201–10. doi: 10.1074/jbc.M910348199
24. Oakley RH, Olivares-Reyes JA, Hudson CC, Flores-Vega F, Dautzenberg FM, Hauger RL. Carboxyl-terminal and intracellular loop sites for CRF1 receptor phosphorylation and β-arrestin-2 recruitment: a mechanism regulating stress and anxiety responses. *Am J Physiol Regul Integr Comp Physiol*. (2007) 293:R209–22. doi: 10.1152/ajpregu.00099.2006
25. Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ. Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. *J Biol Chem*. (1994) 269:6193–7.
26. Servitja JM, Marinissen MJ, Sodhi A, Bustelo XR, Gutkind JS. Rac1 function is required for Src-induced transformation. Evidence of a role for Tiam1 and Vav2 in Rac activation by Src. *J Biol Chem*. (2003) 278:34339–46. doi: 10.1074/jbc.M302960200
27. Hauger RL, Olivares-Reyes JA, Braun S, Catt KJ, Dautzenberg FM. Mediation of corticotropin releasing factor type 1 receptor phosphorylation and desensitization by protein kinase C: a possible role in stress adaptation. *J Pharmacol Exp Ther*. (2003) 306:794–803. doi: 10.1124/jpet.103.050088
28. Shah BH, Olivares-Reyes JA, Catt KJ. The protein kinase C inhibitor Go6976 [12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] potentiates agonist-induced mitogen-activated protein kinase activation through tyrosine phosphorylation of the epidermal growth factor receptor. *Mol Pharmacol*. (2005) 67:184. doi: 10.1124/mol.104.003533
29. Olivares-Reyes J, Shah B, Hernandez-Aranda J, Garcia-Caballero A, Farshori M, Garcia-Sainz J, et al. Agonist-induced interactions between angiotensin AT(1) and epidermal growth factor receptors. *Mol Pharmacol*. (2005) 68:356–64. doi: 10.1124/mol.104.010637
30. Milan-Lobo L, Gsandtner I, Gaubitzer E, Runzler D, Buchmayer F, Kohler G, et al. Subtype-specific differences in corticotropin-releasing factor receptor complexes detected by fluorescence spectroscopy. *Mol Pharmacol*. (2009) 76:1196–210. doi: 10.1124/mol.109.059139
31. Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ. Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J Biol Chem*. (1995) 270:17148–53. doi: 10.1074/jbc.270.29.17148
32. Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem*. (1996) 271:19443–50. doi: 10.1074/jbc.271.32.19443
33. Rozengurt E. Mitogenic signaling pathways induced by G protein-coupled receptors. *J Cell Physiol*. (2007) 213:589–602. doi: 10.1002/jcp.21246
34. Luttrell LM. Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. *J Mol Neurosci*. (2005) 26:253–64. doi: 10.1385/JMN:26:2-3:253
35. Wee P, Wang Z. Epidermal growth factor receptor cell proliferation signaling pathways. *Cancers*. (2017) 9:E52. doi: 10.3390/cancers9050052
36. Wright JD, Reuter CWM, Weber MJ. Identification of sites on epidermal growth factor receptors which are phosphorylated by pp60src *in vitro*. *Biochim Biophys Acta*. (1996) 1312:85–93. doi: 10.1016/0167-4889(96)00027-4
37. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res*. (2003) 284:31–53. doi: 10.1016/S0014-4827(02)00098-8
38. Eguchi S, Dempsey PJ, Frank GD, Motley ED, Inagami T. Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J Biol Chem*. (2001) 276:7957–62. doi: 10.1074/jbc.M008570200
39. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. (1999) 402:884–8. doi: 10.1038/47260
40. Shah BH, Baukal AJ, Shah FB, Catt KJ. Mechanisms of extracellularly regulated kinases 1/2 activation in adrenal glomerulosa cells by lysophosphatidic acid and epidermal growth factor. *Mol Endocrinol*. (2005) 19:2535–48. doi: 10.1210/me.2005-0082
41. Shah B, Yesilkaya A, Olivares-Reyes J, Chen H, Hunyady L, Catt K. Differential pathways of angiotensin II-induced extracellularly regulated kinase 1/2 phosphorylation in specific cell types: role of heparin-binding epidermal growth factor. *Mol Endocrinol*. (2004) 18:2035–48. doi: 10.1210/me.2003-0476
42. Sun Y, McGarrigle D, Huang XY. When a G protein-coupled receptor does not couple to a G protein. *Mol Biosyst*. (2007) 3:849–54. doi: 10.1039/b706343a
43. Luttrell DK, Luttrell LM. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene*. (2004) 23:7969–78. doi: 10.1038/sj.onc.1208162
44. Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature*. (1996) 383:547–50. doi: 10.1038/383547a0
45. Sato K, Sato A, Aoto M, Fukami Y. c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. *Biochem Biophys Res Commun*. (1995) 215:1078–87. doi: 10.1006/bbrc.1995.2574
46. Sato K. Cellular functions regulated by phosphorylation of EGFR on Tyr845. *Int J Mol Sci*. (2013) 14:10761–90. doi: 10.3390/ijms140610761
47. Perkovska S, Mejean C, Ayoub MA, Li J, Hemery F, Corbani M, et al. V1b vasopressin receptor trafficking and signaling: role of arrestins, G proteins and Src kinase. *Traffic*. (2018) 19:58–82. doi: 10.1111/tra.12535
48. Shah BH, Catt KJ. Calcium-independent activation of extracellularly regulated kinases 1 and 2 by angiotensin II in hepatic C9 cells: roles of protein kinase Cdelta, Src/proline-rich tyrosine kinase 2, and epidermal growth receptor trans-activation. *Mol Pharmacol*. (2002) 61:343–51. doi: 10.1124/mol.61.2.343
49. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol*. (2010) 11:329–41. doi: 10.1038/nrm2882
50. Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ. Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem*. (1996) 271:12133–6. doi: 10.1074/jbc.271.21.12133
51. Bisotto S, Fixman ED. Src-family tyrosine kinases, phosphoinositide 3-kinase and Gab1 regulate extracellular signal-regulated kinase 1 activation induced by the type A endothelin-1 G-protein-coupled receptor. *Biochem J*. (2001) 360:77–85. doi: 10.1042/bj3600077
52. Laffargue M, Raynal P, Yart A, Peres C, Wetzker R, Roche S, et al. An epidermal growth factor receptor/Gab1 signaling pathway is required for activation of phosphoinositide 3-kinase by lysophosphatidic acid. *J Biol Chem*. (1999) 274:32835–41. doi: 10.1074/jbc.274.46.32835
53. Yart A, Roche S, Wetzker R, Laffargue M, Tonks N, Mayeux P, et al. A function for phosphoinositide 3-kinase beta lipid products in coupling beta gamma to Ras activation in response to lysophosphatidic acid. *J Biol Chem*. (2002) 277:21167–78. doi: 10.1074/jbc.M110411200
54. Liu L, Xie Y, Lou L. PI3K is required for insulin-stimulated but not EGF-stimulated ERK1/2 activation. *Eur J Cell Biol*. (2006) 85:367–74. doi: 10.1016/j.ejcb.2005.11.005
55. Sampaio C, Dance M, Montagner A, Edouard T, Malet N, Perret B, et al. Signal strength dictates phosphoinositide 3-kinase contribution to Ras/extracellular signal-regulated kinase 1 and 2 activation via differential Gab1/Shp2 recruitment: consequences for resistance to epidermal growth factor receptor inhibition. *Mol Cell Biol*. (2008) 28:587–600. doi: 10.1128/MCB.01318-07
56. Duckworth BC, Cantley LC. Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *J Biol Chem*. (1997) 272:27665–70. doi: 10.1074/jbc.272.44.27665
57. New DC, Wu K, Kwok AW, Wong YH. G protein-coupled receptor-induced Akt activity in cellular proliferation and apoptosis. *FEBS J*. (2007) 274:6025–36. doi: 10.1111/j.1742-4658.2007.06116.x
58. Arcaro A, Aubert M, Espinosa del Hierro ME, Khanzada UK, Angelidou S, Tetley TD, et al. Critical role for lipid raft-associated Src kinases in activation of PI3K-Akt signalling. *Cell Signal*. (2007) 19:1081–92. doi: 10.1016/j.cellsig.2006.12.003

59. Pleiman CM, Hertz WM, Cambier JC. Activation of phosphatidylinositol-3′ kinase by Src-family kinase SH3 binding to the p85 subunit. *Science*. (1994) 263:1609–12. doi: 10.1126/science.8128248
60. Strungs EG, Luttrell LM. Arrestin-dependent activation of ERK and Src family kinases. *Handb Exp Pharmacol*. (2014) 219:225–57. doi: 10.1007/978-3-642-41199-1_12
61. Menard L, Ferguson SS, Zhang J, Lin FT, Lefkowitz RJ, Caron MG, et al. Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta-arrestin determine kinetics of internalization. *Mol Pharmacol*. (1997) 51:800–8. doi: 10.1124/mol.51.5.800
62. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, et al. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science*. (1999) 283:655–61. doi: 10.1126/science.283.5402.655
63. Holmes KD, Babwah AV, Dale LB, Poulter MO, Ferguson SS. Differential regulation of corticotropin releasing factor 1alpha receptor endocytosis and trafficking by beta-arrestins and Rab GTPases. *J Neurochem*. (2006) 96:934–49. doi: 10.1111/j.1471-4159.2005.03603.x
64. Inda C, Armando NG, Dos Santos Claro PA, Silberstein S. Endocrinology and the brain: corticotropin-releasing hormone signaling. *Endocr Connect*. (2017) 6:R99–120. doi: 10.1530/EC-17-0111
65. Papadopoulou N, Chen J, Randeva HS, Levine MA, Hillhouse EW, Grammatopoulos DK. Protein kinase A-induced negative regulation of the corticotropin-releasing hormone R1alpha receptor-extracellularly regulated kinase signal transduction pathway: the critical role of Ser301 for signaling switch and selectivity. *Mol Endocrinol*. (2004) 18:624–39. doi: 10.1210/me.2003-0365
66. Robichaux WG, Cheng X. Intracellular cAMP sensor EPAC: physiology, pathophysiology, and therapeutics development. *Physiol Rev*. (2018) 98:919–1053. doi: 10.1152/physrev.00025.2017
67. Van Kolen K, Dautzenberg FM, Verstraeten K, Royaux I, De Hoogt R, Gutknecht E, et al. Corticotropin releasing factor-induced ERK phosphorylation in AtT20 cells occurs via a cAMP-dependent mechanism requiring EPAC2. *Neuropharmacology*. (2010) 58:135–44. doi: 10.1016/j.neuropharm.2009.06.022
68. Ma YC, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. *Cell*. (2000) 102:635–46. doi: 10.1016/S0092-8674(00)00086-6
69. Gao Y, Tang S, Zhou S, Ware JA. The thromboxane A2 receptor activates mitogen-activated protein kinase *via* protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. *J Pharmacol Exp Ther*. (2001) 296:426–33.
70. Luttrell LM, la Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ. Gbeta gamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J Biol Chem*. (1997) 272:4637–44. doi: 10.1074/jbc.272.7.4637
71. Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors: convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem*. (1997) 272:19125–32. doi: 10.1074/jbc.272.31.19125
72. Wells V, Downward J, Mallucci L. Functional inhibition of PI3K by the [beta]GBP molecule suppresses Ras-MAPK signalling to block cell proliferation. *Oncogene*. (2007) 26:7709–14. doi: 10.1038/sj.onc.1210580
73. Kimura Y, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Darnel AD, et al. Expression of urocortin and corticotropin-releasing factor receptor subtypes in the human heart. *J Clin Endocrinol Metab*. (2002) 87:340–6. doi: 10.1210/jcem.87.1.8160
74. Takahashi K, Totsune K, Murakami O, Saruta M, Nakabayashi M, Suzuki T, et al. Expression of urocortin III/stresscopin in human heart and kidney. *J Clin Endocrinol Metab*. (2004) 89:1897–903. doi: 10.1210/jc.2003-031663
75. Ikeda K, Tojo K, Inada Y, Takada Y, Sakamoto M, Lam M, et al. Regulation of urocortin I and its related peptide urocortin II by inflammatory and oxidative stresses in HL-1 cardiomyocytes. *J Mol Endocrinol*. (2009) 42:479–89. doi: 10.1677/JME-08-0151
76. Ikeda K, Tojo K, Otsubo C, Udagawa T, Hosoya T, Tajima N, et al. Effects of urocortin II on neonatal rat cardiac myocytes and non-myocytes. *Peptides*. (2005) 26:2473–81. doi: 10.1016/j.peptides.2005.05.021
77. Chen-Scarabelli C, Saravolatz L II, McCaukey R, Scarabelli G, Di Rezze J, Mohanty B, et al. The cardioprotective effects of urocortin are mediated *via* activation of the Src tyrosine kinase-STAT3 pathway. *JAK-STAT*. (2013) 2:e24812. doi: 10.4161/jkst.24812
78. Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, et al. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet*. (2000) 24:403–9. doi: 10.1038/74255
79. Paneda C, Winsky-Sommerer R, Boutrel B, de Lecea L. The corticotropin-releasing factor-hypocretin connection: implications in stress response and addiction. *Drug News Perspect*. (2005) 18:250–5. doi: 10.1358/dnp.2005.18.4.908659
80. Pelissier-Rota M, Chartier NT, Bonaz B, Jacquier-Sarlin MR. A crosstalk between muscarinic and CRF2 receptors regulates cellular adhesion properties of human colon cancer cells. *Biochimi Biophys Acta-Mol Cell Res*. (2017) 1864:1246–59. doi: 10.1016/j.bbamcr.2017.04.008
81. Ducarouge B, Pelissier-Rota M, Laine M, Cristina N, Vachez Y, Scoazec JY, et al. CRF2 signaling is a novel regulator of cellular adhesion and migration in colorectal cancer cells. *PLoS ONE*. (2013) 8:e79335. doi: 10.1371/journal.pone.0079335
82. Grisanti LA, Guo S, Tilley DG. Cardiac GPCR-mediated EGFR transactivation: impact and therapeutic implications. *J Cardiovasc Pharmacol*. (2017) 70:3–9. doi: 10.1097/FJC.0000000000000462
83. Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, et al. The β2-adrenergic receptor mediates extracellular signal-regulated kinase activation *via* assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem*. (2000) 275:9572–80. doi: 10.1074/jbc.275.13.9572
84. Tilley DG, Kim IM, Patel PA, Violin JD, Rockman HA. beta-Arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. *J Biol Chem*. (2009) 284:20375–86. doi: 10.1074/jbc.M109.005793
85. Fan G-f, Shumay E, Malbon CC, Wang H-y. c-Src tyrosine kinase binds the β2-adrenergic receptor *via* phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J Biol Chem*. (2001) 276:13240–7. doi: 10.1074/jbc.M011578200
86. Laporte SA, Scott MGH. β-arrestins: multitask scaffolds orchestrating the where and when in cell signalling. In: Scott MGH, Laporte SA, editors. *Beta-Arrestins: Methods and Protocols*. New York, NY: Springer New York (2019). p. 9–55. doi: 10.1007/978-1-4939-9158-7_2
87. Perry SJ, Junger S, Kohout TA, Hoare SR, Struthers RS, Grigoriadis DE, et al. Distinct conformations of the corticotropin releasing factor type 1 receptor adopted following agonist and antagonist binding are differentially regulated. *J Biol Chem*. (2005) 280:11560–8. doi: 10.1074/jbc.M412914200
88. Bonfiglio JJ, Inda C, Senin S, Maccarrone G, Refojo D, Giacomini D, et al. B-Raf and CRHR1 internalization mediate biphasic ERK1/2 activation by CRH in hippocampal HT22 Cells. *Mol Endocrinol*. (2013) 27:491–510. doi: 10.1210/me.2012-1359
89. Dunn HA, Chahal HS, Caetano FA, Holmes KD, Yuan GY, Parikh R, et al. PSD-95 regulates CRFR1 localization, trafficking and β-arrestin2 recruitment. *Cell Signal*. (2016) 28:531–40. doi: 10.1016/j.cellsig.2016.02.013
90. Flood DG, Finn JP, Walton KM, Dionne CA, Contreras PC, Miller MS, et al. Immunolocalization of the mitogen-activated protein kinases p42MAPK and JNK1, and their regulatory kinases MEK1 and MEK4, in adult rat central nervous system. *J Comp Neurol*. (1998) 398:373–92. doi: 10.1002/(SICI)1096-9861(19980831)398:3<373::AID-CNE6>3.0.CO;2-X
91. Ross CA, Wright GE, Resh MD, Pearson RC, Snyder SH. Brain-specific src oncogene mRNA mapped in rat brain by *in situ* hybridization. *Proc Natl Acad Sci USA*. (1988) 85:9831–5. doi: 10.1073/pnas.85.24.9831
92. Sugrue MM, Brugge JS, Marshak DR, Greengard P, Gustafson EL. Immunocytochemical localization of the neuron-specific form of the c-src gene product, pp60c-src(+), in rat brain. *J Neurosci*. (1990) 10:2513–27. doi: 10.1523/JNEUROSCI.10-08-02513.1990

93. Pawlak R, Magarinos AM, Melchor J, McEwen B, Strickland S. Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior. *Nat Neurosci*. (2003) 6:168–74. doi: 10.1038nn998
94. Yang CH, Huang CC, Hsu KS. Behavioral stress modifies hippocampal synaptic plasticity through corticosterone-induced sustained extracellular signal-regulated kinase/mitogen-activated protein kinase activation. *J Neurosci*. (2004) 24:11029–34. doi: 10.1523/JNEUROSCI.3968-04.2004
95. Refojo D, Echenique C, Muller MB, Reul JM, Deussing JM, Wurst W, et al. Corticotropin-releasing hormone activates ERK1/2 MAPK in specific brain areas. *Proc Natl Acad Sci USA*. (2005) 102:6183–8. doi: 10.1073/pnas.0502070102
96. Krishnan V, Han MH, Mazei-Robison M, Iniguez SD, Ables JL, Vialou V, et al. AKT signaling within the ventral tegmental area regulates cellular and behavioral responses to stressful stimuli. *Biol Psychiatry*. (2008) 64:691–700. doi: 10.1016/j.biopsych.2008.06.003
97. Dahlhoff M, Siegmund A, Golub Y, Wolf E, Holsboer F, Wotjak CT. AKT/GSK-3[beta]/[beta]-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms. *Neuroscience*. (2010) 169:1216–26. doi: 10.1016/j.neuroscience.2010.05.066

Conflict of Interest: FD was employed by Novaliq GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 26 June 2020
doi: 10.3389/fendo.2020.00372

A Gq Biased Small Molecule Active at the TSH Receptor

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G protein coupled receptors (GPCRs) can lead to G protein and non-G protein initiated signals. By virtue of its structural property, the TSH receptor (TSHR) has a unique ability to engage different G proteins making it highly amenable to selective signaling. In this study, we describe the identification and characterization of a novel small molecule agonist to the TSHR which induces primary engagement with $G_{\alpha q/11}$. To identify allosteric modulators inducing selective signaling of the TSHR we used a transcriptional-based luciferase assay system with CHO-TSHR cells stably expressing response elements (CRE, NFAT, SRF, or SRE) that were capable of measuring signals emanating from the coupling of $G_{\alpha s}$, $G_{\alpha q/11}$, $G_{\beta\gamma}$, and $G_{\alpha 12/13}$, respectively. Using this system, TSH activated $G_{\alpha s}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$ but not $G_{\beta\gamma}$. On screening a library of 50K molecules at 0.1,1.0 and 10 μM, we identified a novel $G_{q/11}$ agonist (named MSq1) which activated $G_{q/11}$ mediated NFAT-luciferase >4 fold above baseline and had an $EC_{50} = 8.3 \times 10^{-9}$ M with only minor induction of $G_{\alpha s}$ and cAMP. Furthermore, MSq1 is chemically and structurally distinct from any of the previously reported TSHR agonist molecules. Docking studies using a TSHR transmembrane domain (TMD) model indicated that MSq1 had contact points on helices H1, H2, H3, and H7 in the hydrophobic pocket of the TMD and also with the extracellular loops. On co-treatment with TSH, MSq1 suppressed TSH-induced proliferation of thyrocytes in a dose-dependent manner but lacked the intrinsic ability to influence basal thyrocyte proliferation. This unexpected inhibitory property of MSq1 could be blocked in the presence of a PKC inhibitor resulting in derepressing TSH induced protein kinase A (PKA) signals and resulting in the induction of proliferation. Thus, the inhibitory effect of MSq1 on proliferation resided in its capacity to overtly activate protein kinase C (PKC) which in turn suppressed the proliferative signal induced by activation of the predomiant cAMP-PKA pathway of the TSHR. Treatment of rat thyroid cells (FRTL5) with MSq1 did not show any upregulation of gene expression of the key thyroid specific markers such as thyroglobulin(Tg), thyroid peroxidase (Tpo), sodium iodide symporter (Nis), and the TSH receptor (Tshr) further suggesting lack of involvement of MSq1 and

OPEN ACCESS

Edited by:
László Hunyady, Semmelweis University, Hungary

Reviewed by:
Stanko S. Stojilkovic, National Institutes of Health (NIH), United States
Thierry Durroux, Centre National de la Recherche Scientifique (CNRS), France

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Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 January 2020*
Accepted: *11 May 2020*
Published: *26 June 2020*

Citation:
Latif R, Morshed SA, Ma R, Tokat B, Mezei M and Davies TF (2020) A Gq Biased Small Molecule Active at the TSH Receptor. Front. Endocrinol. 11:372. doi: 10.3389/fendo.2020.00372

$G_{\alpha q/11}$ activation with cellular differentation. In summary, we identified and characterized a novel $G_{\alpha q/11}$ agonist molecule acting at the TSHR and which showed a marked anti-proliferative ability. Hence, Gq biased activation of the TSHR is capable of ameliorating the proliferative signals from its orthosteric ligand and may offer a therapeutic option for thyroid growth modulation.

Keywords: TSH, GPCR, gprotein, proliferation, agonist

INTRODUCTION

Traditionally GPCR drug development has focused on conventional agonists and antagonists that are known to act as "on-off" switches. However, there is growing appreciation that GPCRs can mediate their physiologically relevant effects through selective signaling due to subtle structural changes and engagement of G protein and non-G protein effectors. Selective signaling can be driven by endogenous ligands, synthetic peptides or small molecules, which bind to the orthosteric or allosteric site(s) and in turn bias the downstream signal. The TSHR which is made up of a large glycosylated ectodomain and seven transmembrane helices which are connected by extracellular and intracellular loops (1) is structurally poised as a candidate for allosteric modulation with its ability to engage all four classes of G proteins (2). Studies using both modeling and mutational analysis of the TSHR have indicated the structural determinants of the G protein coupling to the receptor (3, 4). However, it is not yet fully clear as to what preferential order these different G proteins are engaged by the TSHR during activation nor the exact intra- and -inter molecular interactions leading to coupling of the different G proteins by TSH or TSHR antibodies. However, crystallization of the partial ectodomain with stimulating and blocking autoantibodies (5–7) together with studies of the molecular rearrangement of the TSHR ectodomain and hinge regions has given some recent insight into the possible mechanism(s) of this activation (8, 9).

Small molecules can bind to the allosteric sites on the TSHR TMD and ectodomain (10, 11) and are excellent tools to gain insight into the potential for TSHR selective signaling. Their unique ability to readily permeate the cell membrane and interact with specific residues within the transmembrane helices can induce subtle conformational changes (12–14). In recent years there has been rapid development of small molecules, both agonists (15–17) and antagonists (16, 18–20) against the TSHR as part of a search for novel therapeutic agents. These various small molecule ligands induce the $G\alpha_s$ pathway of the TSHR and the possibility of selective $G\alpha_{q/11}$ activation by a small molecule has not been explored. However, studies have indicated that such selectivity in signaling can be established in GPCRs and not only by different receptor subtypes (21, 22) but also via pathway bias suggesting ligand selectivity can be a potential source of a defined pharmacology for small molecules (23, 24).

Abbreviations: TSHR, Thyroid stimulating receptor; GPCR, G protein coupled receptors; GD, Graves' disease; TMD, transmembrane domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In this report, we describe the identification and *in vitro* characterization of a novel small molecule that activates the TSHR by preferentially initiating $G\alpha_{q/11}$ signaling and then examined its biological consequences on thyrocyte proliferation and gene expression.

MATERIALS AND METHODS

Establishing Double Transfected CHO-TSHR Cell Lines

In order to identify the signaling through the four major classes of G-proteins ($G_{\alpha s}$,$G_{\alpha q/11}$ and $G_{\alpha 12/13}$ and $G_{\beta\gamma/i}$) by the TSHR, we generated double transfected CHO-TSHR stable lines containing CRE, NFAT, SRF, or SRE response elements (RE) tagged to a modified form of luciferase reporter. These double transfected stable clones were established by selecting the cells with hygromycin (800 ug/ml) and 500 ug/ml of G418 (neomycin sulfate). Following initial screening and validation, these stable cell lines were maintained in Ham's F-12 medium with 10% fetal bovine serum (FBS), 100 units of penicillin and streptomycin with 200 ug/ml of hygromycin and G418 to maintain the selection pressure in these co-transfected cells. Using the individually co-transfected stable lines containing the respective response elements, we screened a 50K chemical library at 0.1,1 and 10 μM against CRE, NFAT, SRF, and SRE cells in a 384 well format following the protocol described previously (17).

Treatment and Lysate Preparation

For downstream signaling studies, low passage number of FRTL5 cells were cultured in 60 mm dishes using Hams F12 medium with 5% calf serum to which 1X 6H (6 hormone mixture) was added as previously described (25). Once the cells reached 60–80% confluence, cells were washed twice with plain medium and then cultured further in Ham F12 medium containing only 5H hormone (-TSH) for 72 hrs. Following this the cells were washed twice with plain F12 medium and incubated for another 48 h in Ham's F12 medium containing 0.3% BSA (basal medium). These cells were then either stimulated with increasing dose of MSq1, TSH or MS438 or combination of TSH plus MSq1 or TSH+MSq1+ PKC inhibitor at 2 μM (G06883) as per the experimental details described under figure legends for 48 h at 37C. Lysates from these treated cells were prepared using 1X Novagen phosphosafe extraction buffer as per the manufacturer's instructions and total protein in the lysate estimated by Bradford (26). Further the proteins were resolved on 4–15% SDS-PAGE and transferred to PVDF membranes by wet transfer and classic immunblotting performed for detection of phospho protein after

blocking membranes with 2% BSA for 2 h at RT or subjected to protein quantification using simple western system by the WES machine for immunoblotting and detection (ProteinSimple, Santa Clara, CA, USA).

Immunoblotting and Detection

In the present study, we quantitated the absolute response to PKC and PKA in the lysate prepared from the treated cells as descried above. pPKC was detected by classical immunoblotting procedure described earlier (27) using commercially obtained primary antibodies to pPKC βII ser660, Anti-rabbit HRP (1:20,000) in 1X tris-borate saline with tween 20 0.5% (TBST) was used as detection antibody and the immunoblots developed with ECL. Quantitation of pPKA was carried out using the protein simple WES system after titrating the primary and secondary antibodies against different concentrations of the samples. Briefly, the WES protocol is as described here, first, a 0.2 μg of lysate was mixed with master mix to achieve a final concentration of 1X sample buffer in the presence of fluorescent molecular weight marker and 40 mM dithiothreitol, the samples were denatured at 95°C for 5 min. Target proteins were immunoprobed with primary antibody pPKA (thr197) followed by HRP-conjugated secondary antibodies. All antibodies were diluted using an antibody diluent at a 1:100 or 1:200. Detection of ERK 44-kDa protein in the lysate using anti ERK served as a positive run control in addition to the biotinylated ladder for size estimation. β-actin was used as the loading control. Digital images of the signal were analyzed with Compass software (ProteinSimple), and the quantified data of the detected proteins with the correct molecular weight is reported as signal/noise ratio derived from average signal intensity exposures.

Proliferation Measured by Alamar Blue

Proliferation of FRTL5 cells was measured using Alamar Blue, which monitors the reducing environment of the living cell. The active ingredient is resazurin, which is a stable, nontoxic and permeable compound, which accepts electrons and changes from the oxidized, non-fluorescent, blue state to the reduced, fluorescent, pink state. These studies were carried out on FRTL5 grown on black clear bottom 96 well plates. Cells in the log phase were harvested by trypsin and seeded as 30×10^3 cells/well and allowed to adhere to the bottom of plate in complete HamF12 medium by incubating the cells with 6H overnight at 37°C. Following a 24 to 36 hrs incubation, the cells were culturally prepared by removing TSH for 3 days prior to induction of proliferation as described earlier. The cells were then exposed to MSq1, TSH or combination of both with and without the PKC inhibitor as per the experiment described under figure legends. For determining the effect of a small molecule or TSH on cell growth, we had stimulated vs. unstimulated cells. Following 48 h of treatment, Alamar Blue was aseptically added to each well in an amount equal to 10% of the volume in the wells. Cells with Alamar Blue were further incubated at 37°C for another 5 h prior to reading the plates. Proliferation was assessed by measuring fluorescence intensity of the reduced dye at 540/580 nm. Wells with media plus dye only was used as the background control. Log change between untreated over that of treated groups was deduced from the fluorescent intensities obtained after background subtraction.

Docking and Contact sites

Docking of the lead MSq1 molecules was performed on a homology model of the TSHR-TMD based on rhodopsin (PDB:1F88). This template was chosen because of the low RMSD values between the backbone of the TM helices of the TSHR model and that of the rhodopsin x-ray crystal structure (14) and fits the experimental parameters that we have previously described (15). The initial homology model of rhodopsin TMD was obtained from the Uniprot server (http://www.uniprot.org). The conformations of the extracellular loops were constructed with a Monte Carlo method (16). The 3D geometries of the docked ligands were generated with MarvinSketch (http://www.chemaxon.com). Multiple docking was carried out using the programs Glide, Autodock-4 and Autodock-Vina. The docking results were analyzed using Dockres and other supporting script tools (17). In particular, Dockres extracts the coordinates of the docked poses from the docking log file and identifies contacts between the ligand and target as pairs of mutually proximal atoms and hydrogen bonds (if any) as X…H-X' where X and X' are polar atoms (one on the ligand and the other on the target) with X…H distance within threshold and X…H-X angle is greater than 120 deg.

IP-One Assay

In principle PLC is the main intracellular effector enzyme of $G_{\alpha q/11}$-coupled GPCRs. PLC hydrolyzes PIP_2 into IP_3 and DAG. The intracellular second messenger IP_3 is rapidly degraded by phosphatases and recycled back via inositol into cell membrane PIP_2. Thus, for measuring $G_{\alpha q/11}$ activation by MSq1 in CHOTSHR cells we used the Cisbio IP-One Gq kit which is a competitive immunoassay intended to measure myo-inositol-1phosphate (IP1) accumulation in cells. The inositol phosphate accumulation assay utilizes the ability of lithium to inhibit the breakdown of inositol monophosphates and detects this accumulated IP1 by HTRF® technology. In the assay native IP1 produced by cells or unlabeled IP1 (standard curve) compete with d2-labeled IP1 (acceptor) for binding to anti-IP1-Cryptate (donor). The specific signal (i.e., energy transfer) is inversely proportional to the concentration of IP1 in the standard or sample. 50×10^3 CHOTSHR cells per well were seeded in 96 well black plates in complete Hams F12 medium and incubated overnight at 37°C. The adherent cells were gently washed once with warm plain medium with low serum (2%) and the cells were treated with increasing doses of TSH (μU) or MSq1 (μM) in stimulation buffer containing 50 mM of lithium chloride. At the end of 2 h incubation the cells were lysed using the lysis buffer provided and treated with detection antibodies as per manufacture's instructions and run along with the standards provided in the kit. The measurement of acceptor (665 nm) to donor (620 nM) emission was obtained using the microplate plate reader ClarioStar and ratio calculated and interpolated to standard curves to calculate the values of the unknown samples.

TSHR Expression by Flow-Cytometry

ML-1 and FT236, two follicular cancer lines, were grown in DMEM high glucose with 10 % FBS, 200 mM glutamine, 1x sodium pyruvate 1X Minimum essential medium with 100 units of penicillin and streptomycin. The cells were detached from the plate non-enzymatically using 1 mM EGTA/EDTA and washed twice with 1X PBS, filtered using 75 micron filter and total cells counted. 0.5×10^6 cells/tube were suspended in 100 ul of FACS staining buffer (1X PBS with 0.2% sodium azide and 2% FBS) with anti TSHR mAb RSR1 mouse Mab (0.1 μg/ml) and incubated for 1 h at room temperature. Following 2x wash with FACS buffer (1XPBS with 0.02% sodium azide) and the bound TSHR receptor antibodies were detected using anti-mouse antibody Fab' phycoerthrin (PE) labeled secondary antibody at 1:200. Unstained cells, isotype antibody or secondary antibody alone were used as controls in the assay. The results were expressed as the percentage positive cells detected in the test samples compared to the controls by the vertical gates assigned based on the controls.

Gene Expression

For gene expression analysis, total RNA was extracted using a RNeasy kit and was treated with ribonuclease-free deoxyribonuclease. Five micrograms of total RNA were reverse transcribed into cDNA using the SuperScript III system. All Q-PCRs was performed using the Step OnePlus Real-time PCR system (Applied Biosystems, Foster City, CA). The reactions were established with 10 μL of SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 μl (2 μM) of sense/anti-sense gene-specific primers, 2 μl of cDNA and DEPC-treated water to a final volume of 20 μl. The PCR reaction mix was denatured at 95°C for 60 s before the first PCR cycle. The thermal cycle profile was used is as follows: denaturizing for 30 s at 95°C; annealing for 30 s at 57–60°C (dependent on primers); and extension for 60 s at 72°C. A total of 40 PCR cycles were used. For each target gene, the relative gene expression was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Data presented as fold change in relative gene expression are from two independent experiments in which all sample sets was analyzed in triplicate.

Statistical Analyses

All curve fitting and P value calculations (one-way ANOVA) were carried using GraphPad Prism 5 software. All assays were performed at least 2 or 3 times as indicated. In case of immunoblot one representative experiment is shown.

RESULTS

Identification of a Unique Gq Activator

In order to identify allosteric ligands that can activate different G proteins of the TSHR we first developed a series of CHO-TSHR cells that were transfected with different response elements tagged to luciferase that can specifically identify the activation of specific G proteins as indicated schematically in **Figure 1A**. The activation of these different response elements was validated using bovine TSH as indicated in **Figure 1B**. This analysis clearly indicated that TSH was capable of activating $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ in a dose-dependent manner. No activation was observed of $G\beta\gamma$ in this system. The respective positive controls used for each of the response elements are indicated and explained in the figure legends.

Screening a 50K library at 0.1,1 and 10 μM against this panel of stable CHO TSHR luciferase cells allowed us to identify a small molecule, which preferentially activated CHO-TSHR-NFAT luciferase cells. Further examination of this Gq activator (named MSq1) against CHOTSHR-NFAT, which couples $G_{\alpha q/11}$, and CHOTSHR-CRE, which measures activation via $G\alpha_s$, in a dose-dependent manner (**Figure 2A**) showed MSq1 to be a potent activator of Gq with an $EC_{50} = 8.3 \times 10^{-9}$M after normalizing the data to max TSH (10^4μU/ml). MSq1 had only minor activation toward Gs thus making this molecule a preferential Gq activator. Structurally this molecule differed from any of the known agonist or antagonist small molecules (**Figure 2B**). Control studies with MSq1 measuring its influence on activation in normal CHO cells (without a TSHR) but transfected with either NFAT luciferase or CRE luciferase at 10 μM showed no activation of luciferase (**Figure S1**). We have shown that MSq1 is incapable of activating either $G_{\beta\gamma}$ or $G_{12/13}$ using the luciferase system further confirming that this is a $G_{q/11}$ biased novel small molecule (**Figure S2**).

Binding Sites of MSq1 by Docking Studies

We examined the binding sites of this Gq activator by *in-silico* docking using the structure of the TSHR TMD region developed by homology modeling and based on the rhodopsin crystal structure (as detailed in Methods). Using the top scoring docking poses generated by Autodock-4 and the criterion of ≤4Å, the putative contact points of MSq1 within the TSHR TMD were deduced. Like most allosteric small molecules against the TSHR, the MSq1 sites were nestled in the "hydrophobic pocket" formed by the different helices within the TSHR TMD (**Figure 3A**). Further analysis indicated that MSq1 made major contact points on the TSHR TMD helices H1, H2, H3, and H7 within the hydrophobic pocket and the extracellular loops including L2-3 & L4-5 (**Figure 3B**). When these contact residues were compared to our $G\alpha_s$ agonist MS438 some overlapping, and some unique residues could be observed as shown in **Table 1** which lists the top-scoring Glide, Autodock-4 and Autodock-Vina poses for both MS438 and MSq1.

Downstream Signaling of the Gq Activator

Since the *in-silico* modeling confirmed the potential binding of MSq1 to the TSHR TMD, we examined the key downstream signals that are known to be driven by Gq activation. Activation of PLC was assayed by measuring IP1 accumulation, which showed that MSq1 and TSH were capable of significantly increasing IP1 generation (**Figure 4A** inset)**.** Furthermore,

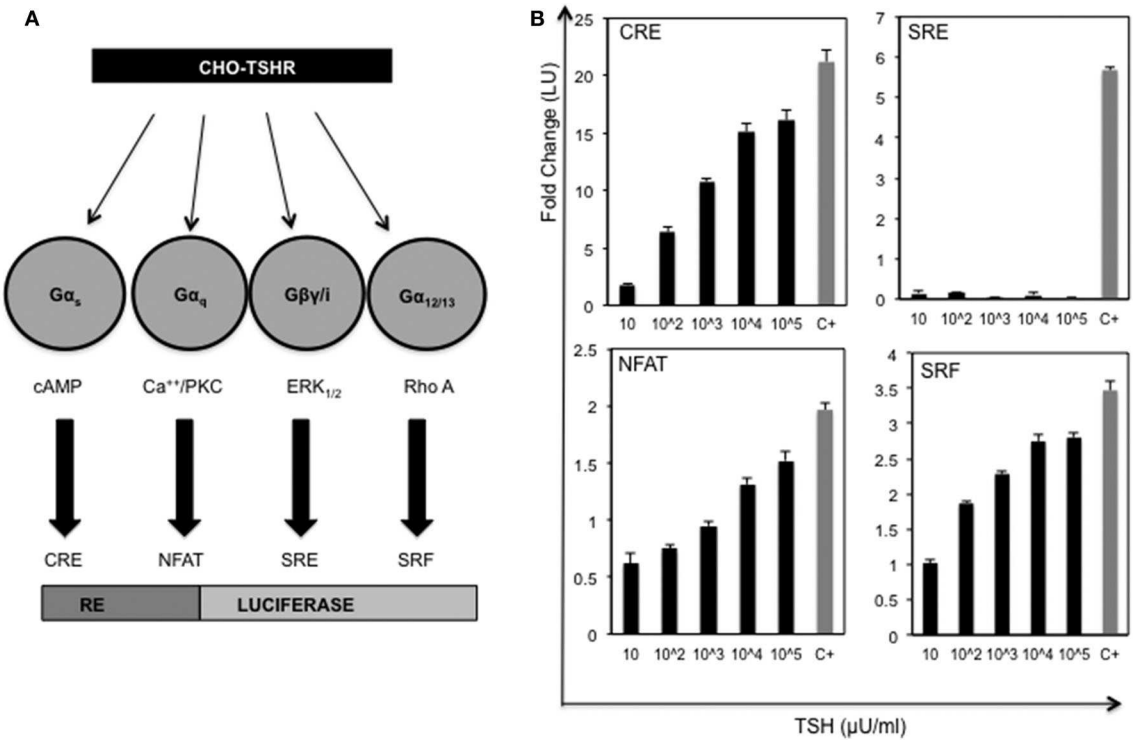

FIGURE 1 | Activation of the TSH receptor and G proteins. **(A)** Schematic representation of double transfected CHO-TSHR cells generated to study activation of the different G proteins. Shown are the 4 luciferase tagged response elements (REs) that are capable of measuring the activation of the respective second messengers used by the G proteins. **(B)** The bar graph panels represent the dose-responses with TSH (10 µU to 10^5 µU/ml) and the respective positive controls used with each of the double transfected stable cells. The change in activity is represented as fold change of luciferase units (LU) on the Y-axis. The gray bars marked with C+ in the x -axis are the positive controls for the each of the response elements. The positive controls used were as follows: CRE - forskolin 5 uM, NFAT- ionomycin 100 µM, SRF- 20% serum + PMA 10 ng and SRE−20% serum. The data represented here are from 3 separate experiments. Note that all the data shown here are baseline subtracted.

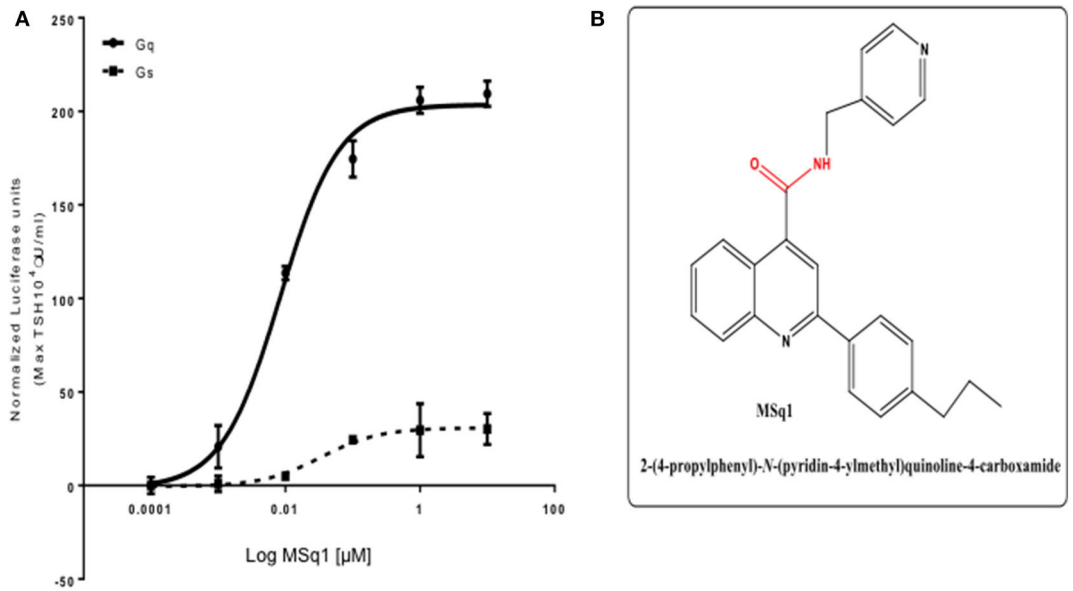

FIGURE 2 | (A) Potency of selected $G\alpha_{q/11}$ agonist MSq1. Dose-response relationship of MSq1 against the NFAT and CRE response element containing cells. A 4-fold increase of NFAT luciferase was observed with MSq1 compared to a small increase in CRE luciferase showing that MSq1 is biased toward $G\alpha_{q/11}$ signaling. **(B)** Molecular structure of MSq1. Chemical structure and SMILE of Msq1. The blue represents the nitrogen atoms and red corresponds to the oxygen atoms in a carbon backbone. Chemically it is 2-(4-propylphenyl)-N-(4-pyridinylmethyl)-4-quinolinecarboxamide. Name:C1(C2=CC=CC=C2N=C(C2C=CC(=CC=2) CCC) C=1) C(=O)NCC1C=CN=CC=1.

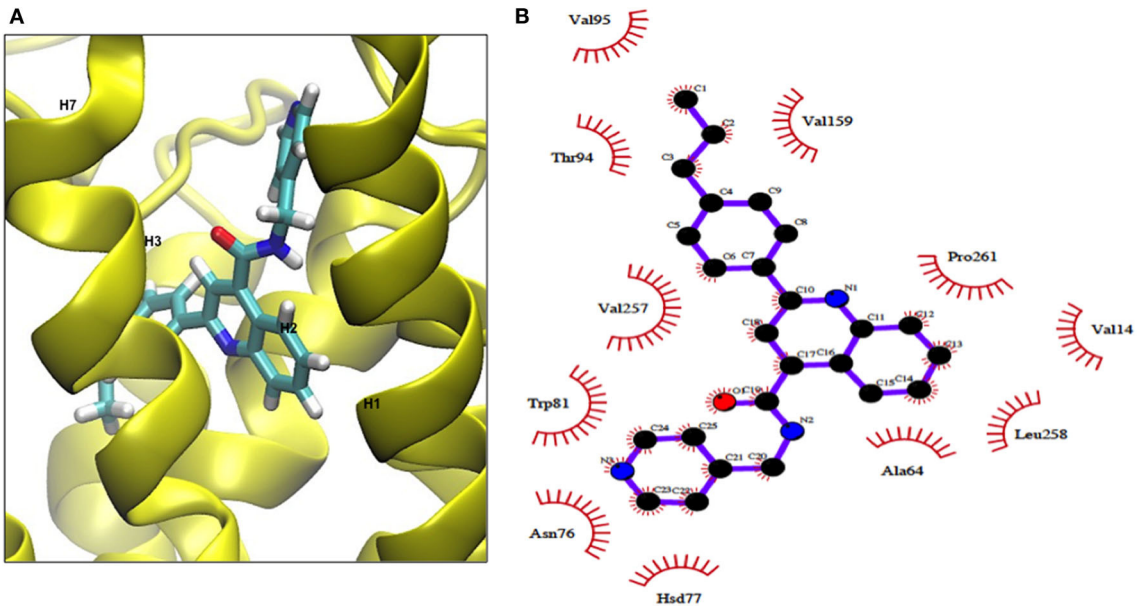

FIGURE 3 | (A) Binding of MSq1 molecule to the TSHR TMD. A homology model of the TSHR transmembrane domain, previously described (28), was used as the template for docking studies. Analysis of the Autodock results as detailed in Materials and Methods indicated that MSq1, like other small molecules, docks into a hydrophobic pocket of the TSHR TMD and in this case makes contact with residues in helices H1, H2, H3, H6, and H7 and the extracellular loops 2,3 and 4,5. **(B)** The TSHR TMD and its contact sites with MSq1. On extracting co-ordinates of the docked poses using Dockres, the program showed contact resides against the TSHR TMD (red semi asterisks) assessed by the criteria of $\leq$ 4Å as indicated in this diagram. Furthermore, these contact residues in the TSHR TMD and their location within the TMD residues are indicated along with the contacts for MS438 in **Table 1** for comparison.

TABLE 1 | TSHR residues on the TMD that MSq1 and MS438 contact.

			MS438			MSq1		
TSHR residue	**Residue No #**	**Ballest #**	**rG(l-P)**	**rA(L-P)**	**rV(l-p)**	**rG(L-P)**	**rA(L-P)**	**rV(L-P)**
LEU	10	1.35		3.1	3.6		3.1	
VAL	14	1.39			3.2			3.4
VAL	17	1.42			3.4			
LEU	60	2.57		3.2	3.2			3.2
LEU	61	2.58		3.2	3.1			
ALA	64	2.57	2.9	2.8	3.5	2.9	2.8	
ASN	76	L (2-3)	3.1	2.8	3.6	2.9		
TRP	81	L (2-3)			3.8	3.1		
CYS	87	3.25	3.6			3.6		
ALA	90	3.28	4.0		3.7	4.0		
GLY	91	3.29	3.0		3.6			3.6
THR	94	3.32		2.8	2.7	3.8	3.5	3.7
VAL	95	3.33		3.1	3.6		3.1	3.6
SER	98	3.36		3.3	3.4		3.4	3.4
GLU	99	3.37			3.7			
LYS	158	L (4-5)		3.2				
VAL	159	L (4-5)		3.4	3.5	3.7		4.1
ILE	233	6.51		3.2	3.4			3.4
LYS	253	7.42		3.3	3.8		3.3	
ILE	254	7.43		3.3				
VAL	257	7.46	3.7	2.7	3.6	3.2	3.3	3.4
LEU	258	7.47		3.1	3.2		3.5	3.4
TYR	260	7.49		3.2			3.5	
PRO	261	7.50	3.1	3.0	3.6	3.1		3.7

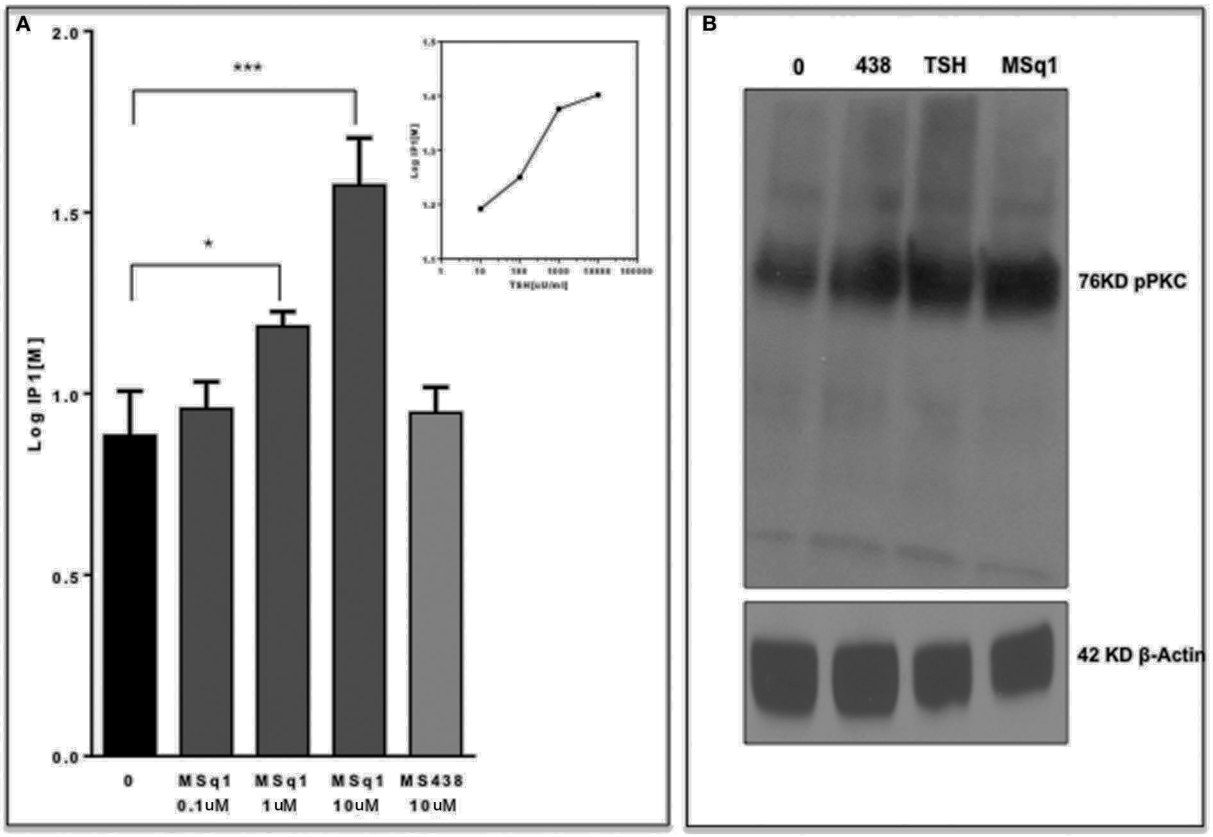

FIGURE 4 | Gq signaling by MSq1. **(A)** Since Gq activation is known to result in an IP1 increase via PLC-β activation, we measured IP activation in CHO-TSHR cells with MSq1 at 0.1 and 10 μM. As indicated here MSq1 showed a significant increase ($P = 0.03$) in IP1 on stimulation with MSq1 which was not observed by MS438 even at 10 uM. The inset shows the dose dependent increase in IP1 with TSH. The data is plotted after background subtraction. **(B)** Total lysates of FRTL5 cells treated with MS438 10 μM, TSH 1,000 μU/mL and MSq1 10 uM for 24 h and the immunoblots probed for pPKC. MSq1 increased pPKC when compared to the unstimulated cells (lane 0). The 42KD β actin was used as the loading control (*$P < 0.05$, ***$P < 0.0001$).

using phospho-specific antibodies against PKC, we observed that MSq1 significantly enhanced pPKC compared to both TSH and MS438 in thyroid (FRTL5) cells (**Figure 4B**, upper panel). However, no significant enhancement of pERK or pAKT was observed by MSq1 activation (**Figure 4B**, lower panel). These downstream signaling studies indicated that MSq1 had the ability to activate the two major arms of $G\alpha_{q/11}$ signaling as shown by NFAT-luciferase activation and enhanced PKC activation.

Inhibition of TSH Induced Proliferation by $G_{\alpha q/11}$ Activation

The physiological significance of cAMP signaling by $G\alpha_s$ coupling on thyrocyte growth and proliferation is well-established. Since the effect of $G\alpha_{q/11}$ on thyroid cell proliferation is not clear we examined the action of MSq1 on proliferation of thyrocytes using rat FRTL5 cells. As indicated in **Figure 5A**, MSq1 failed to enhance basal proliferation of thyrocytes while one of our previously published TSHR agonists (MS438) showed a dose-dependent increase in proliferation and which is known to activate the cAMP-PKA pathway like TSH. In contrast, in the presence of 10^4 μU/ml of TSH, MSq1 inhibited the TSH induced proliferation of thyrocytes in a dose-dependent manner suggesting a suppressive action of Gq activation on the proliferative capacity of the TSH induced Gs-cAMP-PKA pathway (**Figure 5B**). This inhibition was only observed in TSH dependent thyrocytes and ML-1 cells derived from a human follicular carcinoma line with a high expression of TSHRs (75% expression of cell surface TSHR as established by flow cytometry) (**Figure 6A**) or FTC 236 cells, another follicular carcinoma line which totally lacks cell surface TSHR (**Figure 6B**), did not respond to MSq1 actions (**Figures 6C,D**). Examining gene expression for common thyroid differentiation markers such sodium iodide symporter (NIS), thyroglobulin (Tg) and the TSHR by qPCR, we did not find these markers to be upregulated in treated cells, suggesting that MSq1 activation of $G\alpha_{q/11}$ lacked the ability to affect thyrocyte differentiation markers (**Figure S3**).

Release of Inhibitory Effect on Proliferation by PKC Inhibition

In order to examine the mechanism of the suppression of TSH induced thyroid cell proliferation we used a broad-spectrum

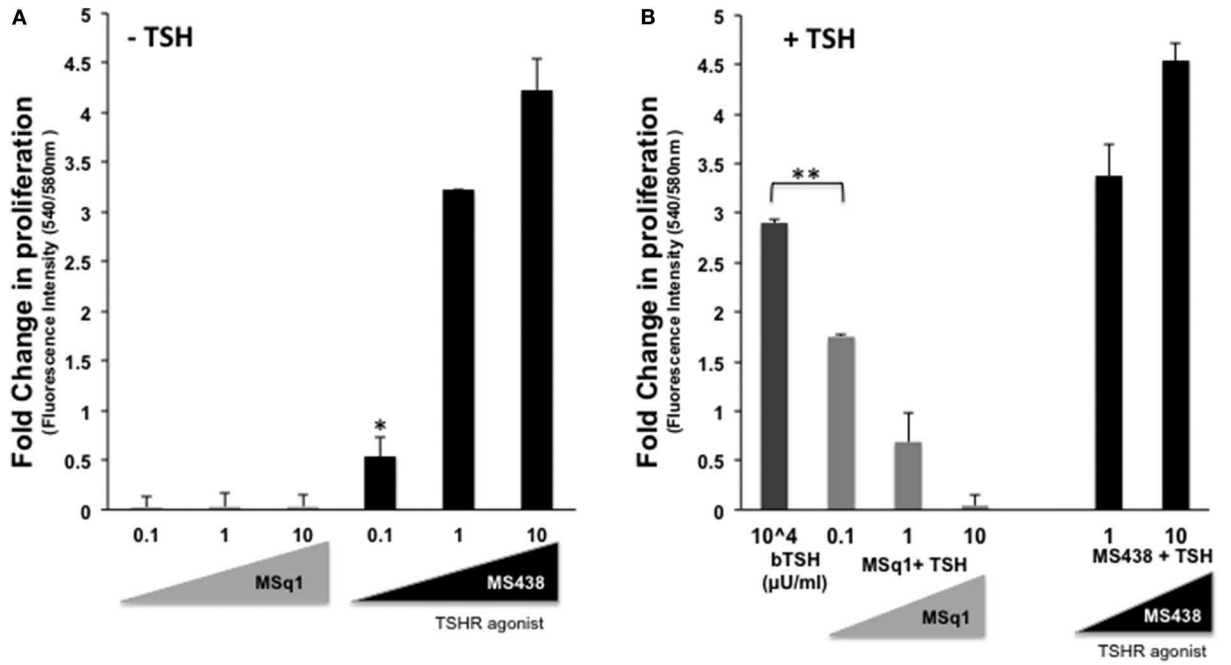

FIGURE 5 | Inhibition of thyrocyte proliferation by MSq1. **(A)** Rat thyrocytes seeded at $30X10^3$/well of 96 well clear bottom black plate and prepared as detailed in materials and method were stimulated with increasing concentrations of MSq1 or MS438. After 48 h of stimulation in basal medium, 1/10th volume of Alamar Blue was added and incubated further at 37°C for 5 h and fluorescence emitted by the dye was measured. As seen here, MSq1 lacked the ability to stimulate proliferation of thyrocytes in comparison to MS438. **(B)** Similar experiments were performed under the same protocol as described above and in the presence of 10^4 µU/mL of TSH. MSq1, indicated by the light gray bars, showed significant dose-dependent inhibition of TSH-induced proliferation. However, this inhibition was specific to MSq1 since MS438, in the presence of TSH, did not inhibit proliferation but rather enhanced TSH induced proliferation at 1 and 10 µM as indicated by black bars ($n = 3$ $^*P < 0.05$, $^{**}P < 0.001$).

PKC inhibitor (G06983) in the presence of TSH and MSq1. As shown earlier, MSq1 treatment at 10 µM caused inhibition of TSH induced proliferation. However, in the presence of the PKC inhibitor, inhibition of proliferation by MSq1 was markedly reduced (**Figure 7A**). On quantitating the PKA signal using Western blotting with an anti PKA antibody, we observed that cells treated with TSH and MSq1 in the presence of the PKC inhibitor for 48 h showed significantly enhanced PKA signals compared to MSq1 plus TSH or TSH alone (**Figures 7B,C**). These data demonstrated that enhancement of the PKC signal by MSq1 inhibited the cAMP-PKA pathway induced by TSH activation in the thyrocytes.

DISCUSSION

TSH is known to induce engagement of all four classes of G protein (2) with the TSHR. However, the major pathway activated by TSH is the Gαs pathway via PKA (29). The consequence of changing this selection is not well-understood. In particular, the role of the $G\alpha_{q/11}$ pathway via PKC has not been clearly clarified and it is unclear whether overt activation of this pathway has any cellular consequences. Therefore, identifying selective allosteric activators, which are biased to activating a G protein class, is one way of studying the mechanism of TSHR selective signaling and its physiological or pathophysiological effects on thyroid and extra thyroidal TSHRs. This is especially so when knock-out mouse models, which although a very valuable research tool for studying gene function, have their limitations in terms of producing an observable change and may even produce unexpected characteristics which in certain situations cannot be extrapolated to humans (30). In this report, we present data on the identification of a potent $G\alpha_{q/11}$ activator against the TSHR and our examination of its effects on thyrocytes.

In recent years high-throughput screening assays, combined with *in silico* structural approaches and medicinal manipulations, have resulted in the identification of a number of specific and potent agonists (16, 17, 31) and antagonists (18, 19, 32) against the TSHR which effectively activate or inhibit Gαs initiated signals such as the cAMP-PKA pathway. Using a "tool kit" of CHO-TSHR cells harboring CRE, NFAT, SRF or SRE response elements tagged to luciferase, as shown in **Figure 1A,** we identified potent and specific $G\alpha_{q/11}$ selective small molecules. Our search found a molecule (MSq1) unlike our previously reported (17) agonist molecules which is biased toward $G\alpha_{q/11}$. TSH activates predominantly $G\alpha_s$ (33) and $G\alpha_{q/11}$ when used in high (non-physiologic) concentrations (34, 35). Coupling of $G\alpha_{q/11}$ to the TSHR leads to activation of phospholipase C (PLC) which in turn triggers the release of intracellular calcium [Ca^{2+}], and NFAT and alternatively activates protein kinase C (PKC) and its downstream effector

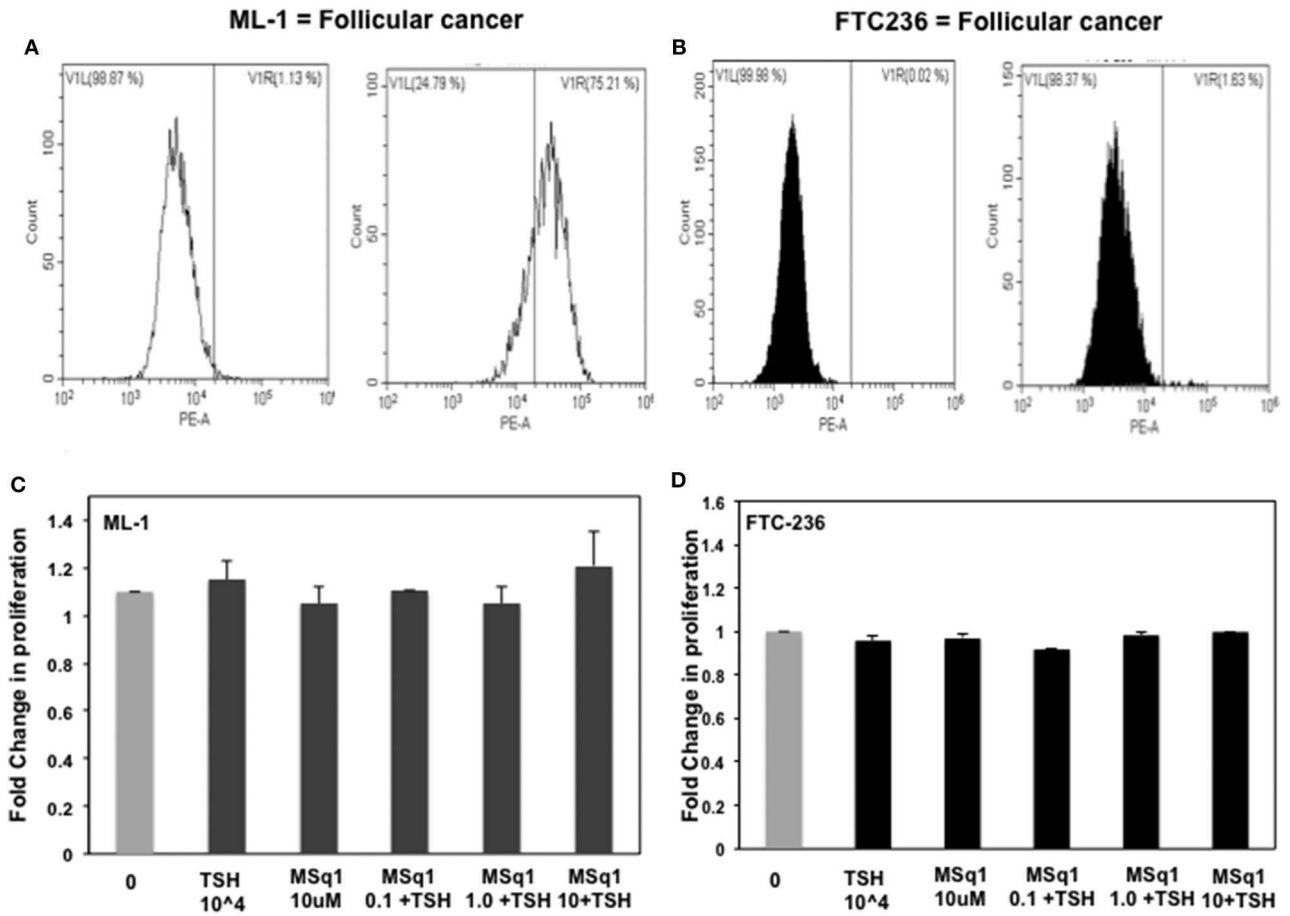

FIGURE 6 | Lack of human thyrocyte proliferation: To ascertain if this inhibition was specific to TSH induced proliferation, we further measured proliferation in two human thyroid follicular carcinoma cell lines with TSH and MSq1. ML-1 cells which are not TSH dependent and express TSHR **(A)** did not show any induction nor inhibition of proliferation **(C)** and likewise FTC236 which lacks TSHR **(B)** also showed no effects on treating with MSq1 **(D)**. These data indicated that the inhibition by MSq1 is observed only by TSH dependent thyroid cells and is not due to any non-specific activation.

MAP kinase (MAPK). The normal physiological consequences of activating $G\alpha_s$ in thyrocytes are proliferation, hormone synthesis and thyroglobulin (Tg) iodination (29, 36). However, the physiological or pathophysiological control of $G\alpha_{q/11}$ signaling in thyrocytes or extrathyroidal TSHRs is not well-characterized despite multiple reports. For example, conditional deletion of $G\alpha_{q/11}$ in mouse thyroid resulted in hypoplastic thyroid glands and severe hypothyroidism (34). It has also been shown that $G\alpha_{q/11}$-PKC dependent activation in TSHR transfected papillary cancer cells (line FTC236) resulted in the upregulation of a class of redox and metal ion scavengers which are cysteine-rich proteins known as metallothioneins (MTs) (37). Studies have also shown an indirect relationship of $G\alpha_{q/11}$ activation to thyroid peroxidase formation (34, 38) and a congenital hypothyroidism phenotype (39).

Our docking studies with a modeled TMD (28) showed that the $G\alpha_{q/11}$ activator molecule binds within the hydrophobic pocket of the TMD. By this analysis we saw that in addition to overlapping contacts with our agonist MS438 ($G\alpha_s$ dominant), the MSq1 molecule also made contact with some unique resides helping to explain its selective allosteric $G\alpha_{q/11}$ activation (**Table 1**). Furthermore, docking MSq1 to the TSH binding surface of the ECD resulted in docking scores that were more than 4 kcal/mol weaker than the top scores observed when docked to the TMD. Such differences represent ∼786 times weaker binding indicating that this molecule, like our previously reported small molecules, is can be a allosteric molecule (17).

Despite tremendous progress into the molecular mechanism concerning contacts and activation of G proteins by GPCRs (40, 41) our understanding as to how structurally distinct ligands may lead to the stabilization of different "active states" of the receptors remains open. Homology modeling of the TSHR with the G_q heterodimer combined with mutational analysis of the transmembrane domain has indicated the principal determinants leading to the complex interaction (4, 14, 42) suggesting spatial conformation for selective G protein activation.

In this study, we observed that MSq1 is an activator of PLC (**Figure 4A**) and its downstream effectors—PKC and NFAT activation (**Figure 4B**). MSq1 showed increased phosphorylation of PKC. However, we failed to see any up regulation in the

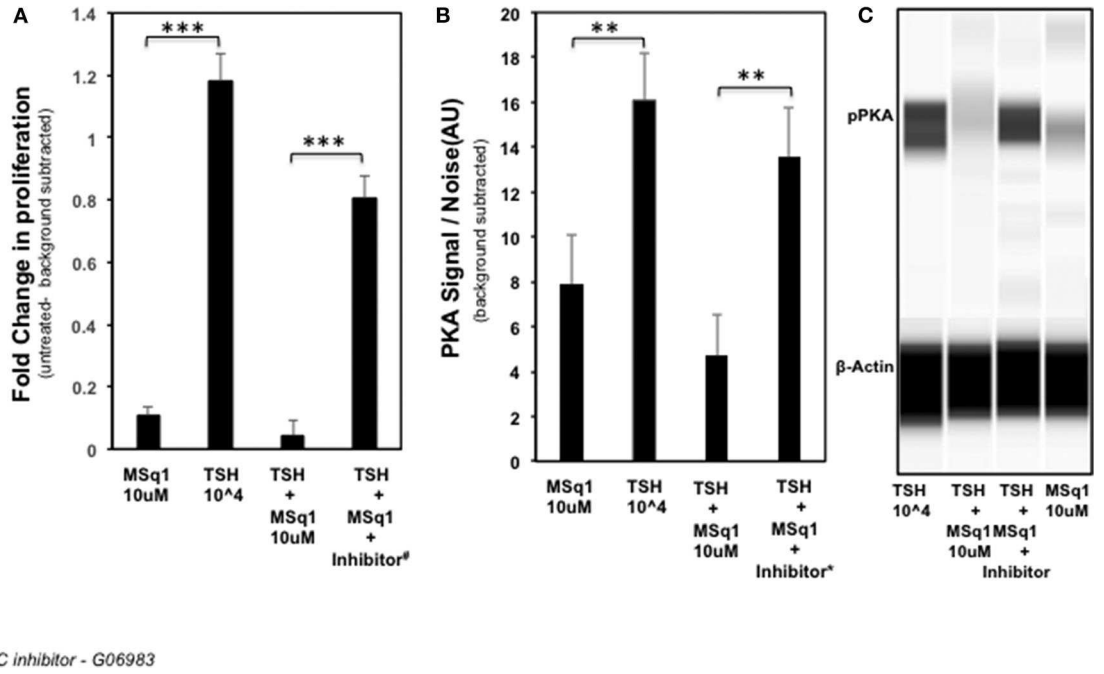

FIGURE 7 | Mechanistic studies on inhibition of thyrocyte proliferation. In order to examine if PKC activation by MSq1 was leading to inhibition of TSH induced proliferation we used a PKC inhibitor (*G06983)*. **(A)** FRTL5 cells were stimulated by MSq1 alone, TSH 10^4 μU/ml alone, TSH +MSq1 and TSH+ MSq1 + PKC inhibitor for 48 h as indicated. MSq1 effectively inhibited TSH induced proliferation but in the presence of this PKC inhibitor (2 uM); proliferation was restored in these cells. Note that the untreated cell data were subtracted as background from all the data (***$P < 0.0001$). **(B)** Since the possible mechanism of this inhibition was likely to be the result of PKA suppression by the induced PKC, we quantitated the levels of pPKA in the treated samples using immunoblotting in the Wes system. As observed here, PKA was significantly decreased with MSq1 as opposed to TSH alone. This suppression of PKA was released when the cells were treated with TSH and MSq1 in the presence of the PKC inhibitor. The untreated cell data were subtracted as background from all the data (***$P < 0.001$). **(C)** The lane representations of the WES output are similar to the data that is represented in panel B and shown here with phosphor PKA and β actin bands.

mRNA levels of thyroid specific genes in contrast to the effect of TSH or our small molecule agonist MS438. On examining the proliferation of these cells, MSq1 alone did not induce any proliferation as seen with MS438 or TSH. It is generally accepted that the proliferation of thyroid cells by TSH is mediated in large part by the cAMP-PKA pathway (43, 44). In contrast, the MSq1 molecule showed the unique ability to suppress TSH induced proliferation. Since we did not observe any blockade of TSH induced cAMP by MSq1 (**Figure S4**) we hypothesized that suppression must be due to interference with the cAMP-PKA pathway and most likely by PKC activation. There exists cross-talk in downstream signaling of GPCRs (45) and it has been previously shown that PKC can suppress PKA induced activation (46) and functional interference between cAMP/PKA and PKC pathways is possible (47, 48). Thus, experiments carried out in the presence of a PKC inhibitor confirmed that inhibiting PKC in the presence of MSq1 and TSH showed a marked reduction in the suppressive effect of MSq1 on proliferation. Furthermore, pPKA levels showed a significant increase after exposure to the PKC inhibitor. The only study which supports a physiological role for the $G\alpha_{q/11}$ mediated signaling pathway in TSH induced hormone synthesis (34) was performed in $G\alpha_{q/11}$ knock out mice. However, the action of MSq1 on proliferation is opposite to the $G\alpha_{q/11}$ study. Our model would suggest that overt activation of the cAMP-PKA pathway by high concentrations of TSH leading to increased proliferation might be kept in check by the PLC-PKC pathway via Gq and thus maintain a balance in the endogenous proliferative capacity of thyrocytes differing with data that contradicts much of the literature which suggested that TSH stimulates differentiation and not proliferation of normal human thyrocytes (49).

In conclusion, we have identified a novel $G\alpha_{q/11}$ biased modulator of the TSHR with inhibitory effects on thyrocyte proliferation. The data illustrate the intertwining molecular mechanisms leading to this action. This raises the prospect of modulating biased TSHR signaling for more specific pharmacologic responses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RL responsible for design, execution of the experiments, data analysis, and manuscript writing. SM helped in the experiments

with western blots. RM helped in PCR experiments. BT helping in carrying out some facs experiments. MM did the modeling and *in-silco* docking studies. TD helped in data analysis and finalizing of the manuscript.

FUNDING

This work was supported in part by National Institute of Health (NIH) grant DK069713, the Segal Family Endowment, and the Veterans Administration Merit Award Program (to TD).

ACKNOWLEDGMENTS

We thank Dr. Bhasker Das from the Departments of Medicine and Pharmacological Sciences, Icahn School of Medicine at Mount Sinai for critical reading of our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00372/full#supplementary-material

REFERENCES

1. Davies TF, Ando T, Lin RY, Tomer Y, Latif R. Thyrotropin receptor-associated diseases: from adenomata to Graves disease. *J Clin Invest.* (2005) 115:1972–83. doi: 10.1172/JCI26031
2. Laugwitz KL, Allgeier A, Offermanns S, Spicher K, van Sande J, Dumont JE, et al. The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci USA.* (1996) 93:116–20. doi: 10.1073/pnas.93.1.116
3. Neumann S, Krause G, Claus M, Paschke R. Structural determinants for g protein activation and selectivity in the second intracellular loop of the thyrotropin receptor. *Endocrinology.* (2005) 146:477–85. doi: 10.1210/en.2004-1045
4. Kleinau G, Jaeschke H, Worth CL, Mueller S, Gonzalez J, Paschke R, et al. Principles and determinants of G-protein coupling by the rhodopsin-like thyrotropin receptor. *PLoS One.* (2010) 5:e9745. doi: 10.1371/journal.pone.0009745
5. Furmaniak J, Sanders J, Nunez Miguel R, Rees Smith B. Mechanisms of Action of TSHR Autoantibodies. *Horm Metab Res.* (2015) 47:735–52. doi: 10.1055/s-0035-1559648
6. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, et al. Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. *Thyroid.* (2007) 17:395–410. doi: 10.1089/thy.2007.0034
7. Sanders P, Young S, Sanders J, Kabelis K, Baker S, Sullivan A, et al. Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *J Mol Endocrinol.* (2011) 46:81–99. doi: 10.1530/JME-10-0127
8. Schaarschmidt J, Huth S, Meier R, Paschke R, Jaeschke H. Influence of the hinge region and its adjacent domains on binding and signaling patterns of the thyrotropin and follitropin receptor. *PLoS One.* (2014) 9:e111570. doi: 10.1371/journal.pone.0111570
9. Schaarschmidt J, Nagel MB, Huth S, Jaeschke H, Moretti R, Hintze V, et al. Rearrangement of the extracellular domain/Extracellular loop 1 interface is critical for thyrotropin receptor activation. *J Biol Chem.* (2016) 291:14095–108. doi: 10.1074/jbc.M115.709659
10. Marcinkowski P, Kreuchwig A, Mendieta S, Hoyer I, Witte F, Furkert J, et al. Thyrotropin receptor: allosteric modulators illuminate intramolecular signaling mechanisms at the interface of ecto- and transmembrane domain. *Mol Pharmacol.* (2019) 96:452–62. doi: 10.1124/mol.119.116947
11. Haas AK, Kleinau G, Hoyer I, Neumann S, Furkert J, Rutz C, et al. Mutations that silence constitutive signaling activity in the allosteric ligand-binding site of the thyrotropin receptor. *Cell Mol Life Sci.* (2011) 68:159–67. doi: 10.1007/s00018-010-0451-2
12. Wenzel-Seifert K, Seifert R. Molecular analysis of beta(2)-adrenoceptor coupling to G(s)-, G(i)-, and G(q)-proteins. *Mol Pharmacol.* (2000) 58:954–66. doi: 10.1124/mol.58.5.954
13. Moller S, Vilo J, Croning MD. Prediction of the coupling specificity of G protein coupled receptors to their G proteins. *Bioinformatics.* (2001) 17(Suppl. 1):S174–81. doi: 10.1093/bioinformatics/17.suppl_1.S174
14. Kleinau G, Haas AK, Neumann S, Worth CL, Hoyer I, Furkert J, et al. Signaling-sensitive amino acids surround the allosteric ligand binding site of the thyrotropin receptor. *FASEB J.* (2010) 24:2347–54. doi: 10.1096/fj.09-149146
15. Neumann S, Padia U, Cullen MJ, Eliseeva E, Nir EA, Place RF, et al. An enantiomer of an oral small-molecule TSH receptor agonist exhibits improved pharmacologic properties. *Front Endocrinol (Lausanne).* (2016) 7:105. doi: 10.3389/fendo.2016.00105
16. Neumann S, Gershengorn MC. Small molecule TSHR agonists and antagonists. *Ann Endocrinol (Paris).* (2011) 72:74–6. doi: 10.1016/j.ando.2011.03.002
17. Latif R, Ali MR, Ma R, David M, Morshed SA, Ohlmeyer M, et al. New small molecule agonists to the thyrotropin receptor. *Thyroid.* (2015) 25:51–62. doi: 10.1089/thy.2014.0119
18. Neumann S, Eliseeva E, McCoy JG, Napolitano G, Giuliani C, Monaco F, et al. A new small-molecule antagonist inhibits Graves' disease antibody activation of the TSH receptor. *J Clin Endocrinol Metab.* (2011) 96:548–54. doi: 10.1210/jc.2010-1935
19. Marcinkowski P, Hoyer I, Specker E, Furkert J, Rutz C, Neuenschwander M, et al. A new highly thyrotropin receptor-selective small-molecule antagonist with potential for the treatment of graves' orbitopathy. *Thyroid.* (2019) 29:111–23. doi: 10.1089/thy.2018.0349
20. Latif R, Realubit RB, Karan C, Mezei M, Davies TF. TSH receptor signaling abrogation by a novel small molecule. *Front Endocrinol (Lausanne).* (2016) 7:130. doi: 10.3389/fendo.2016.00130
21. Luttrell LM, Maudsley S, Bohn LM. Fulfilling the promise of "Biased" G protein-coupled receptor agonism. *Mol Pharmacol.* (2015) 88:579–88. doi: 10.1124/mol.115.099630
22. Ulloa-Aguirre A, Reiter E, Crepieux P. FSH receptor signaling: complexity of interactions and signal diversity. *Endocrinology.* (2018) 159:3020–35. doi: 10.1210/en.2018-00452
23. White KL, Scopton AP, Rives ML, Bikbulatov RV, Polepally PR, Brown PJ, et al. Identification of novel functionally selective kappa-opioid receptor scaffolds. *Mol Pharmacol.* (2014) 85:83–90. doi: 10.1124/mol.113.089649
24. Tschammer N, Bollinger S, Kenakin T, Gmeiner P. Histidine 6.55 is a major determinant of ligand-biased signaling in dopamine D2L receptor. *Mol Pharmacol.* (2011) 79:575–85. doi: 10.1124/mol.110.068106
25. Morshed SA, Ma R, Latif R, Davies TF. How one TSH receptor antibody induces thyrocyte proliferation while another induces apoptosis. *J Autoimmun.* (2013) 47:17–24. doi: 10.1016/j.jaut.2013.07.009
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3
27. Morshed SA, Latif R, Davies TF. Characterization of thyrotropin receptor antibody-induced signaling cascades. *Endocrinology.* (2009) 150:519–29. doi: 10.1210/en.2008-0878
28. Ali MR, Latif R, Davies TF, Mezei M. Monte Carlo loop refinement and virtual screening of the thyroid-stimulating hormone receptor transmembrane domain. *J Biomol Struct Dyn.* (2015) 33:1140–52. doi: 10.1080/07391102.2014.932310
29. Vassart G, Dumont JE. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr Rev.* (1992) 13:596–611. doi: 10.1210/er.13.3.596

30. Davey RA, MacLean HE. Current and future approaches using genetically modified mice in endocrine research. *Am J Physiol Endocrinol Metab.* (2006) 291:E429–38. doi: 10.1152/ajpendo.00124.2006
31. Neumann S, Huang W, Titus S, Krause G, Kleinau G, Alberobello AT, et al. Small-molecule agonists for the thyrotropin receptor stimulate thyroid function in human thyrocytes and mice. *Proc Natl Acad Sci USA.* (2009) 106:12471–6. doi: 10.1073/pnas.0904506106
32. Neumann S, Pope A, Geras-Raaka E, Raaka BM, Bahn RS, Gershengorn MC. A drug-like antagonist inhibits thyrotropin receptor-mediated stimulation of cAMP production in Graves' orbital fibroblasts. *Thyroid.* (2012) 22:839–43. doi: 10.1089/thy.2011.0520
33. Laurent E, Mockel J, van Sande J, Graff I, Dumont JE. Dual activation by thyrotropin of the phospholipase C and cyclic AMP cascades in human thyroid. *Mol Cell Endocrinol.* (1987) 52:273–8. doi: 10.1016/0303-7207(87)90055-4
34. Kero J, Ahmed K, Wettschureck N, Tunaru S, Wintermantel T, Greiner E, et al. Thyrocyte-specific Gq/G11 deficiency impairs thyroid function and prevents goiter development. *J Clin Invest.* (2007) 117:2399–407. doi: 10.1172/JCI 30380
35. Song Y, Massart C, Chico-Galdo V, Jin L, De Maertelaer V, Decoster C, et al. Species specific thyroid signal transduction: conserved physiology, divergent mechanisms. *Mol Cell Endocrinol.* (2010) 319:56–62. doi: 10.1016/j.mce.2010.01.024
36. De Felice M, Postiglione MP, Di Lauro R. Minireview: thyrotropin receptor signaling in development and differentiation of the thyroid gland: insights from mouse models and human diseases. *Endocrinology.* (2004) 145:4062–7. doi: 10.1210/en.2004-0501
37. Back CM, Stohr S, Schafer EA, Biebermann H, Boekhoff I, Breit A, et al. TSH induces metallothionein 1 in thyrocytes via Gq/11- and PKC-dependent signaling. *J Mol Endocrinol.* (2013) 51:79–90. doi: 10.1530/JME-12-0200
38. Song Y, Driessens N, Costa M, De Deken X, Detours V, Corvilain B, et al. Roles of hydrogen peroxide in thyroid physiology and disease. *J Clin Endocrinol Metab.* (2007) 92:3764–73. doi: 10.1210/jc.2007-0660
39. Garcia M, Gonzalez de Buitrago J, Jimenez-Roses M, Pardo L, Hinkle PM, Moreno JC. Central hypothyroidism due to a TRHR mutation causing impaired ligand affinity and transactivation of Gq. *J Clin Endocrinol Metab.* (2017) 102:2433–42. doi: 10.1210/jc.2016-3977
40. Mahoney JP, Sunahara RK. Mechanistic insights into GPCR-G protein interactions. *Curr Opin Struct Biol.* (2016) 41:247–54. doi: 10.1016/j.sbi.2016.11.005
41. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature.* (2011) 477:549–55. doi: 10.1038/nature10361
42. Kleinau G, Neumann S, Gruters A, Krude H, Biebermann H. Novel insights on thyroid-stimulating hormone receptor signal transduction. *Endocr Rev.* (2013) 34:691–724. doi: 10.1210/er.2012-1072
43. Jin S, Hornicek FJ, Neylan D, Zakarija M, McKenzie JM. Evidence that adenosine 3',5'-monophosphate mediates stimulation of thyroid growth in FRTL5 cells. *Endocrinology.* (1986) 119:802–10. doi: 10.1210/endo-119-2-802
44. Tramontano D, Moses AC, Veneziani BM, Ingbar SH. Adenosine 3',5'-monophosphate mediates both the mitogenic effect of thyrotropin and its ability to amplify the response to insulin-like growth factor I in FRTL5 cells. *Endocrinology.* (1988) 122:127–32. doi: 10.1210/endo-122-1-127
45. Hur EM, Kim KT. G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. *Cell Signal.* (2002) 14:397–405. doi: 10.1016/S0898-6568(01)00258-3
46. Lesage GD, Marucci L, Alvaro D, Glaser SS, Benedetti A, Marzioni M, et al. Insulin inhibits secretin-induced ductal secretion by activation of PKC alpha and inhibition of PKA activity. *Hepatology.* (2002) 36:641–51. doi: 10.1053/jhep.2002.35537
47. Laglia G, Zeiger MA, Leipricht A, Caturegli P, Levine MA, Kohn LD, et al. Increased cyclic adenosine 3',5'-monophosphate inhibits G protein-coupled activation of phospholipase C in rat FRTL-5 thyroid cells. *Endocrinology.* (1996) 137:3170–6. doi: 10.1210/endo.137.8.8754735
48. Sho KM, Okajima F, Abdul Majid M, Kondo Y. Reciprocal modulation of thyrotropin actions by P1-purinergic agonists in FRTL-5 thyroid cells. Inhibition of cAMP pathway and stimulation of phospholipase C-Ca2+ pathway. *J Biol Chem.* (1991) 266:12180–4.
49. Morgan SJ, Neumann S, Marcus-Samuels B, Gershengorn MC. Thyrotropin stimulates differentiation not proliferation of normal human thyrocytes in culture. *Front Endocrinol (Lausanne).* (2016) 7:168. doi: 10.3389/fendo.2016.00168

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